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1) Larson, B., Lipids in Human Saliva, Archs Oral Biol 41(1): 105-110 (1996).

2) Slomiany et al., J. Dent. Res. 61(1): 24-27 (1983).

THanks a bunch!

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LIPIDS IN HUMAN SALIVA

B. LARSSON,[1] G. OLIVECRONA[2] and T. ERICSON[1]

[1]Department of Cariology, and [2]Department of Medical Biochemistry and Biophysics, University of Umeå, 901 87 Umeå, Sweden

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Summary—A simple and reproducible method of determining the quality and quantity of neutral lipids in human saliva was tested. Parotid, submandibular and whole stimulated saliva were collected from 10 healthy adults. The lipids were extracted by the Folch method. A special method for extraction of glycolipids was also tested but gave no additional recovery. Thin-layer chromatography was used for separating the different lipid classes. The concentrations of total lipids in parotid, submandibular and whole stimulated saliva were 0.2, 0.9 and 1.3 mg/dl, respectively. Cholesteryl esters, cholesterol, triglycerides, diglycerides, monoglycerides and free fatty acids accounted for 96–99% of the total salivary lipids. Thus, polar lipids such as phospholipids contributed only a minor fraction, indicating that the lipids are not primarily of membrane origin. Ultracentrifugation of saliva samples at $d = 1.21\ g\ ml^{-1}$ showed that the salivary lipids did not float like blood plasma lipoproteins. Therefore, they must be in a different state of aggregation from lipids in blood or lymph. No significant lipase activity of the type that acts on plasma lipoproteins was found in parotid or submandibular saliva. The content of free fatty acids and partial glycerides was high.

Key words: lipids, saliva.

INTRODUCTION

The presence of lipids in saliva has been known since the work of Doubleday (1909). However, compared to the extensive amount of work on other salivary constituents, e.g. proteins and glycoproteins, rather few studies have been published on lipids of salivary gland origin (Doubleday, 1909; Weber, 1930; Krasnow, 1934; Krasnow and Rosen, 1936; Dirksen, 1963; Mandel and Einstein, 1969; Rabinowitz and Shannon, 1975; Dirksen, 1979; Slomiany, Slomiany and Mandel, 1980; Alam and Alam, 1982; Slomiany *et al.*, 1986). Their concerted finding is that neutral lipids dominate, constituting approx. 70–95% of the total lipids in saliva. The neutral lipids consist of cholesterol, cholesteryl esters, tri-, di- and monoglycerides and free fatty acids (Mandel and Einstein, 1969; Rabinowitz and Shannon, 1975; Dirksen, 1979; Slomiany, Slomiany and Mandel, 1980). The polar lipids consist of different phospholipids, e.g. phosphatidylcholine and phosphatidylethanolamine, and of glycolipids, e.g. cerebrosides (Mandel and Enstein, 1969; Rabinowitz and Shannon, 1975; Dirksen, 1979; Slomiany, Slomiany and Mandel, 1980).

There are few quantitative studies of salivary lipids, probably because only small amounts are present. Most such studies have focused on a particular class of lipids, or even on particular fatty acids. Mandel and Einstein (1969) were the first to make a quantitative assay of the total salivary lipids; they reported a lipid content of 2.0 mg/100 ml of saliva from the submaxillary gland and 2.8 mg/100 ml of saliva from the parotid; 5% of these were phospholipids. Rabinowitz and Shannon (1975) found 6.9 mg/100 ml parotid saliva; phospholipids contributed 30% of the total lipids. Slomiany, Slomiany and Mandel (1980) reported a total lipid content of 8.0 mg/100 ml in submaxillary saliva and 7.6 mg/100 ml parotid saliva; they found that glycolipids amounted to 25–30% and phospholipids to 2–5% of the total lipids. The large discrepancies in the results from different investigators are probably due to differences in methodology, both in collection of saliva and the analytical techniques. In the early reports the methods for saliva collection and the populations studied were often incompletely described. A mixture of alcohol–diethyl ether was usually used for lipid extraction. Lipid quantitation was mostly synonymous with measurements of organic phosphorus. In more recent studies the Folch extraction method with chloroform and methanol has been used in combination with different kinds of separation methods, e.g. thin-layer (Rabinowitz and Shannon, 1975), column (Slomiany, Slomiany and Mandel, 1980) or gas chromatography (Alam 1975; Slomiany *et al.*, 1990).

No published attempt has been made to investigate the origin of the lipids or their physical state in saliva. Neither are there any data on the function of these lipids or on how they might be turned over. The presence of salivary lipases is known, but it was concluded that they have a very limited effect on lipid metabolism in the mouth (Pritchard *et al.*, 1967).

Our aim now was to investigate neutral lipids in human saliva and to establish their concentrations.

Abbreviation: TLC, thin-layer chromatography.

MATERIALS AND METHODS

Participants

Ten healthy individuals (one man and nine w men) w rking at a Dental Health Care Service in northern Sweden participated in the study. Their teeth were free from caries lesions and n ne had any signs of active periodontal disease or gingivitis. The age varied between 27 and 53 years, with a mean ± SD of 40.2 ± 9.4.

Saliva collection

All participants fasted for at least 4 h before the collection of stimulated parotid, submandibular and of whole saliva. Parotid saliva was collected via Lashley cups after gustatory stimulation by SST tablets (Salix Pharma AB, Stockholm). Secretion of submandibular saliva was also stimulated by SST tablets; parotid saliva was prevented from entering the mouth with cotton rolls, the sublingual papillae were isolated from the rest of the mouth with individually fitted plastic devices and the submandibular saliva was collected with a Pasteur pipette. Secretion of whole saliva was stimulated by chewing 1 g of paraffin. The first millilitre was discarded, and then the samples were collected in ice-chilled tubes, centrifuged for 30 min at 10,000 *g* and the supernatant saved. After collection, all samples were subdivided for the different analyses and were frozen at −20°C. For ultracentrifugation, fresh samples of saliva were used.

Lipid analyses

For lipid analyses, samples of 20 ml saliva (for neutral lipids) and 70 ml saliva (for polar lipids) were lyophilized in glass tubes. Control experiments showed that the recovery of lipids was the same whether or not the saliva had been mixed with 5 mM EDTA (pH 6.3) and dialysed against water before lyophilization. Therefore, in these studies EDTA and dialysis was omitted. Before the lipid extraction the dried samples were rehydrated in 0.15 ml water for the neutral lipids and 0.45 ml water for the polar lipids. The extraction, done in the original glass tube, was according to Folch, Lees and Stanley (1957), with chloroform/methanol (2:1, v/v), using 6 ml for the neutral lipid samples and 18 ml for the polar lipid samples. The extraction resulted in a two-phase system of which the upper phase containing methanol, water and water-soluble substances was saved for further analysis (see below). The lower phase, containing chloroform and the lipids, was concentrated to near dryness under a stream of nitrogen. The different lipid classes were separated by TLC. The samples were dissolved in 50 μl chloroform and quantitatively placed on a TLC plate (20 × 20 cm) with silica gel as adsorbent and $CaSO_4$ as binder (J. T. Baker Chemical Co., Phillipsburg, NJ). For separation of neutral lipids the plate was developed in choloroform/methanol/acetic acid (98:6:0.1, v/v/v) until the front had reached 3 cm from the top of the plate. After drying in air the plate was redeveloped in hexane/diethyl ether/acetic acid (94:6:0.2, v/v/v) all the way to the top, according to Bitman, Wood and Ruth (1981). For further studies of the polar lipids the silica gel from the application areas, and from the first centimetre of the lanes (the 70-ml saliva sample) where the polar lipids remained, was scraped off into glass tubes. The gel was thoroughly suspended in 6 ml chloroform/methanol (1:1, v/v) to release the polar lipids. After a brief centrifugation at 5000 rev/min the clear supernatant was collected and the silica gel at the bottom of the tube was re-extracted with chloroform/methanol (1:1, v/v) and centrifuged. The two chloroform/methanol extracts were pooled and concentrated under a stream of nitrogen. The samples were dissolved in 50 μl chloroform/methanol and placed on the same kind of TLC plate as for the neutral lipids. The plate was developed close to the top with acetone/hexane (1:3, v/v). After drying it was developed, again to the top, in chloroform/methanol/acetic acid/water (25:15:4:1, v/v/v/v).

Standards of neutral and polar lipids were run on each plate. Cholesterol and cholesteryl esters (cholesteryl oleate) were obtained from Larodan Fine Chemicals, Malmö, Sweden. Triglycerides were from a Folch extraction of the synthetic lipid emulsion Intralipid 10% (Kabi-Pharmacia Parenterals, Stockholm Sweden). Free fatty acid standard (oleic acid) was from Sigma Chemical Co., St Louis, MO, U.S.A. No standards for di- and monoglycerides were used, but the nature of these spots was deduced from comparison of their migration rates with the original article on this method (Bitman and Wood, 1981). Standards for the polar lipids were from Larodan. One contained equal amounts of phosphatidylethanolamine, phosphatidylcholine and lysolecithin (Mix 50) and the other equal amounts of cerebrosides, sulphatides and spingomyelin (Mix 51).

The spots were visualized and quantified according to the method of Bitman and Wood (1981). For this, the plates were dipped for 3 s in a solution of 3% cupric acetate in 8% aqueous phosphoric acid and then drained for 2 min. The back of the plates were wiped dry before they were placed in an oven at 130°C for 30 min. The spots were then quantified by reflection scanning densitometry at 350 nm using a scanning densitometer (Model CS 9000; Shimadzu Scientific Instruments, Inc., Columbia, MD). A calibration curve for each standard was made. The amounts of lipid in the samples were estimated from comparison with the standard curves. No standards were used for the di- and monoglycerides. It was known from the original article (Bitman and Wood, 1981) that the tri- and diglycerides yielded the same colour intensity and that the monoglycerides yielded 27% of the colour intensity of cholesterol when equal amounts of lipid (by weight) were compared.

Control of the upper phase

Control experiments were done on the upper phase, the methanol/water phase, saved from the Folch extraction (Folch, Lees and Stanley, 1957). The methanol/water phase was concentrated and placed on the same kind of TLC plate as used above (Baker). Development, both for neutral and for polar lipids separately (see above), and visualization (Bitman and Wood, 1981) of the plates revealed no detectable spots.

Phospholipid recovery

A radiolabelled phosph lipid, (L-α-dioleoyl-1-[^{14}C]glycerophosph choline (NEN Research Products, MA, U.S.A.) was placed on a TLC plate, which was devel ped as f r the neutral lipids (see ab ve). The silica gel at the starting point and the first centimetre of the lane was scraped into a glass tube and was then extracted twice as described above for the polar lipids. The extracted sample and the remaining silica gel were counted in a Rack Beta scintillation counter (LKB Wallac, Box 776, Sollentuna, Sweden).

Reproducibility

A test for zig-zag scanning reproducibility was made by scanning a single lane with a spot from each of the different standards. The spots were scanned 10 times. The coefficient of variation was 1.0%, a figure close to the result from Bitman and Wood (1981). The reproducibility from both spotting and scanning was determined by zig-zag scanning 10 lanes that had each been spotted with the same amount of standard. The coefficient of variation was 11.4%, which was also close to the 11.2% reported by Bitman and Wood (1981).

Alternative extraction methods

In some previous studies on salivary lipids, fairly high amounts of glycolipids had been reported. To avoid the possibility that they might disappear in the methanol/water phase because of their higher polarity, an alternative extraction method was tested (Carter and Kanfer, 1975). For this, chloroform/methanol (2:1, v/v) was added to the samples. This s lution was then extracted with a 0.2 M glycine–HCl buffer, pH 2.2, containing 100 mM $CaCl_2$ and centrifuged. The upper phase was removed and discarded. This procedure was repeated three times. Chloroform and methanol (1:1, v/v) was added, resulting in a volume ratio of 2:1 (v/v) of chloroform:methanol. This mixture was extracted once with 0.1 M EDTA, pH 7.0, and then four times with a solution of chloroform–methanol–0.1 M EDTA (pH 7.0), 3:48:47 (v/v/v). The phases were separated by centrifugation and the upper phases were saved. Chloroform–methanol (1:1, v/v) was then added to the pooled upper phases and this mixture was concentrated to dryness in a SpeedVac (Savant Instrument Inc., 110–103 Bi-County, Boullevard Farming, Dale, NY, U.S.A.). Chloroform–methanol (1:1, ml) was added and after mixing the solution was passed through a fine-porosity filter (Scott Duran Por 100; Labora AB, Box 726, Upplands Väsby, Sweden). The clear filtrate was then concentrated under nitrogen and placed on a TLC plate for polar lipid analyses.

Plaque collection

In order to measure lipase activity in dental plaques, the participants were instructed to omit oral hygiene n the buccal surfaces of the upper right molars for 3 days. After mouth rinsing with water the teeth were scraped lightly with a curette. The collected plaque, 100 mg, was suspended in 1.5 ml of a buffer (+4°C, pH 8.2) with 1% Triton X-100, 0.1% SOS and protease inhibitors (leupeptin, pepstatin, trasylol, EDTA and heparin), which was previously sh wn to effectively solubilize and stabilize lipoprotein lipase (Bengtss n-Olivecrona and Olivecrona, 1992). The samples were then briefly centrifuged at 2800 *g* and the supernatant phases were analysed for lipase activity.

Lipase measurement

Lipase activities in three different salivary samples and the solubilized plaque samples were analysed using two assays that were developed for lipoprotein lipase and for hepatic lipase, respectively (Bengtsson-Olivecrona and Olivecrona, 1992). One contained a commercial phospholipid-stabilized triglyceride emulsion (Intralipid 10%; Kabi-Pharmacia Parenterals, Stockholm, Sweden) to which [^{3}H]trioleoylglycerol had been added. The other substrate emulsion was trioleoylglycerol stabilized in gum arabic. Both assays were done at pH 8.5 in the presence of albumin as fatty acid acceptor. The sample volumes were 50 μl for saliva and 10 μl for the solubilized plaques. Inoculation was for 100 min at 25°C. The free fatty acids were extracted and counted by liquid scintillation (Bengtsson-Olivecrona and Olivecrona, 1992).

Ultracentrifugation

To investigate whether, like plasma lipoproteins, the salivary lipids could be floated by ultracentrifugation, samples of 4 ml fresh parotid and submandibular saliva were centrifuged, after KBr had been added to reach a density of $d = 1.21\ g\ ml^{-1}$ for 48 h at 10°C in a Beckman SW-60 rotor at 45,000 rev/min (Havel, Eder and Brydon, 1955). The tubes were sliced (with a Beckman tube slicer; Beckman, Box 65, 161 26 Bromma, Sweden) so that a top layer of 300 μl was separated from the remaining subphase. For analyses by TLC, subphases from two tubes were combined, dialysed against water and lyophilized. The same volume of the original saliva (before centrifugation) was dialysed and lyophilized. These samples were then separated on TLC as described above for the neutral lipids. As a control, total lipoproteins from human plasma, isolated by ultracentrifugation at $d = 1.21\ g\ ml^{-1}$, were added to samples of parotid and submandibular saliva, respectively. Final concentrations of triglycerides and of total cholesterol were 0.9 and 90 mg/100 ml, respectively, as determined by enzymatic, colorimetric assay kits (Boehringer Mannheim, Germany). These samples were then centrifuged at $d = 1.21\ g\ ml^{-1}$ as described above. The tubes were sliced and the lipid contents of top layers and subphases determined after dialysis of the samples.

RESULTS

The concentrations of lipids in saliva samples were low, but control experiments showed that, in spite of this, a single extraction of lyophilized samples with chloroform/methanol gave a good recovery. No additi nal lipids were f und by TLC after a sec nd extraction f the ly philized saliva. The recovery of [^{14}C]phosphatidylcholine fr m scraping the applicati n spot ff the TLC plate after development of neutral lipids was better than 94%. We did not find any additi nal polar lipids when the alternative

extraction method (Carter and Kanfer, 1975) was used. On c mparing the two different extraction methods on several portions ($n = 10$) of the same sample, the coefficient of variation was 8.5%.

The total amount of lipids in stimulated parotid, submandibular and whole saliva was 0.21 mg/100 ml, 0.91 mg/100 ml and 1.36 mg/100 ml, respectively. Figures 1 and 2 show the TLC plates and Tables 1 and 2 show the amounts of different neutral and polar lipids, respectively. Neutral lipids were the predominant form. In parotid saliva almost all extracted lipids were non-polar (more than 99%). Submandibular saliva also had a high non-polar content (approx. 98%). In whole saliva the polar lipid content was somewhat higher (3.6%). The neutral lipids consisted of cholesteryl esters, cholesterol, protonated free fatty acids, tri- di- and monoglycerides. In parotid saliva we did not find any tri- and diglycerides. Phosphatidylcholine, phosphatidyle thanolamine, and sulphatides were the identified polar lipids. On the TLC plate for polar lipid analyses there were also some unidentified spots.

Ultracentrifugation

Ultracentrifugation of parotid saliva at $d = 1.21\ g\ ml^{-1}$ for 48 h, which would float all classes of blood plasma lipoproteins, did not cause flotation of the salivary lipids. The infranatants from the centrifuged samples showed the same spots on TLC plates as the sample of saliva that had not been centrifuged. To investigate whether the viscosity of the saliva prevented flotation of lipoproteins, isolated human serum lipoproteins were added to saliva from the parotid and submandibular glands and the samples were then centrifuged for 48 h. Lipid analyses showed more than 98% of the triglycerides and approx. 95% of the cholesterol had floated to the top of the tubes both for parotid and submandibular saliva. Thus, flotation of normal plasma lipoproteins was not impaired by the saliva constituents.

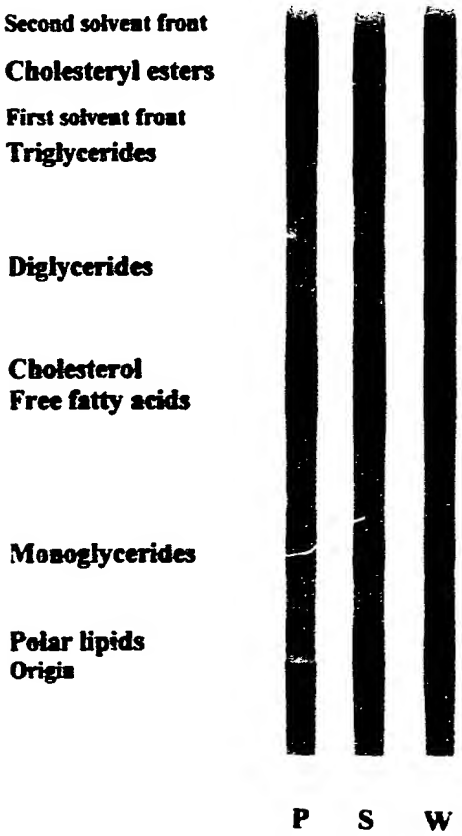

Fig. 1. Part of a TLC plate: 20 ml of parotid (P), submandibular (S) and whole (W) saliva developed for non-polar lipids. The spots of the standards are represented by the text labels.

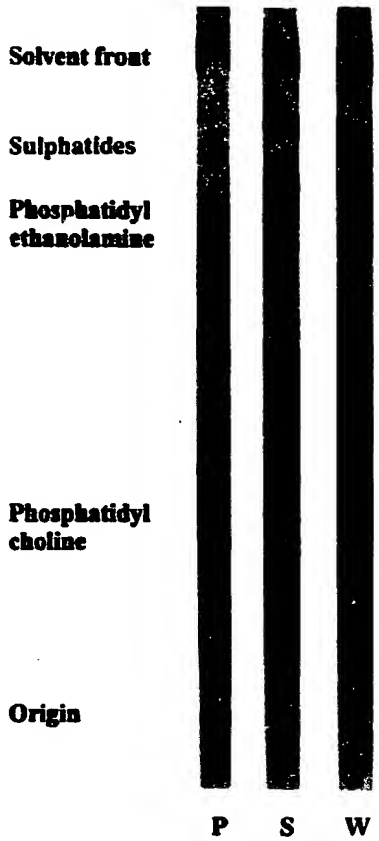

Fig. 2. Part of a TLC plate: 70 ml of parotid (P), submandibular (S) and whole (W) saliva developed for polar lipids. The spots of the standards are represented by the text labels.

Table 1. Non-polar lipids in human saliva from different glands, pooled from 10 individuals (mg/100 ml)

	Parotid saliva	Submandibular saliva	Whole saliva
Cholesteryl esters	0.03	0.26	0.43
Triglycerides	0	0.19	0.29
Diglycerides	0	0.05	0.26
Free fatty acids	0.02	0.07	0.10
Cholesterol	0.09	0.17	0.13
Monoglycerides	0.07	0.15	0.10
Total	0.21	0.89	1.31

Table 2. Polar lipids as percentages of the total amount of polar lipids in parotid, submandibular and whole saliva

	Parotid saliva	Submandibular saliva	Whole saliva
Phosphatidylcholine	14	9	1
Phosphatidylethanolamine	15	23	9
Sulphatides	8	28	7
Unidentified lipids	63	40	78
Percent of non-polar lipids	0.8	2.0	3.6

Lipase activity

The lipase activities in the salivary samples were close to zero with both types of substrate emulsions. Though samples of parotid saliva and of solubilized plaques were incubated with the lipid emulsions for 100 min, no significant lipase activity was found. It must be concluded that these samples did not contain any of the two typical lipases that act on plasma lipoproteins.

DISCUSSION

We have made a qualitative and quantitative analysis of lipids in simulated parotid, submandibular and whole saliva with a simple and reproducible TLC method. Because the lipid concentration of saliva is low, this method has the advantages that the number of manipulations are low, the recovery is good and the lipids are directly visible. For a complete characterization of all lipid classes, more standards and probably also other analytical techniques are required. This was, however, not the aim of the present study. We found a total lipid concentration of 0.21 mg/100 ml in parotid, 0.89 mg/100 ml in submandibular and 1.31 mg/100 ml in whole saliva.

Like previous investigators we found high relative amounts of neutral lipids. In comparison with the total amount of lipids, neutral lipids accounted for 99% in parotid, 98% in submandibular and 96.4% in whole saliva. These figures are close to the results reported by others (Mandel and Einstein, 1969; Krasnow, 1945). However, some investigators report a higher polar lipid content (Rabinowitz and Shannon, 1975; Slomiany *et al.*, 1980). The amount of phospholipids found by Slomiany *et al.* was in agreement with our findings, but they also report a glycolipid concentration of 25–30%. We therefore used a special method for extraction of glycolipids (Carter and Kanfer, 1975), but did not find any more polar lipids than with the regular extraction method. We also analysed the aqueous phase from the Folch extraction method but did not find any additional lipids.

The neutral lipid classes were cholesteryl esters, cholesterol, triglycerides, diglycerides, free fatty acids and monoglycerides; these were in agreement with those reported by others (Mandel and Enstein, 1969; Rabinowitz and Shannon, 1975; Slomiany *et al.*, 1980). Among the polar lipids we could identify phosphatidylch line, phosphatidylethan lamine and sulphatides. As polar lipids are membrane c nstituents, the differences in the polar lipid concentration between whole and submandibular saliva as compared to parotid saliva could be due to a greater contamination of membrane fragments.

The greatest fraction of lipids, at least in whole and submandibular saliva, contained both ch lesteryl esters and glyceryl esters; these are not found in bi logical membranes. The origin of the salivary lipids is not known at the cellular level and the physical state of these lipids has not been studied. As they are practically insoluble in water, they must be complexed with a lipid carrier to be able to exist in saliva. Apart from intracellular lipid droplets in cells like adipocytes and hepatocytes, the only example in the body of dispersed non-polar lipids is provided by the plasma lipoproteins (Mahley *et al.*, 1984). They consist of a core of neutral lipids covered by a layer of phospholipids, cholesterol and specific proteins. The smallest lipoprotein particles of low and high density penetrate the vessel wall and are found in the interstitial fluid. There was a possibility that, for example, high-density lipoproteins or lipoprotein particles of similar composition were secreted by the salivary glands. However, based on our finding that salivary lipids did not float like plasma lipoproteins, we must conclude tha: the salivary lipids are aggregated in a different manner. Moreover, neither saliva nor plaques contained significant amounts of lipases of the type that act on plasma lipoproteins. Still about 10% (w/w) of the neutral lipids were present as free fatty acids.

Slomiany *et al.* (1990) showed by gel-permeation chromatography of saliva that the main part of the neutral lipids appeared to be associated with salivary proteins and glycoproteins. The combined information from their study and from our present ultracentrifugations suggests that saliva contains specifically dispersed lipid–protein complexes. The nature of these complexes and their possible function in the mouth still remain to be established.

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Association of Lipids with Proteins and Glycoproteins in Human Saliva

B. L. SLOMIANY,* H. WITAS,* V. L. N. MURTY,* A. SLOMIANY,* and I. D. MANDEL†

Gastroenterology Research Laboratory, Department of Medicine, New York Medical College, Research Center, Metropolitan Hospital, New York, New York 10029, and †Division of Preventive Dentistry, School of Dental and Oral Surgery, Columbia University, New York, New York 10032

The distribution of lipids in the fractions of parotid and submandibular saliva following Bio-Gel A-50 column chromatography was measured. Over 50% of the total lipids of submandibular saliva was found in the fraction which contained mainly the high-molecular-weight glycoprotein. This fraction also contained most of the glycolipids, free fatty acids, phospholipids, and cholesterol. In the parotid saliva, the fraction containing the basic glycoprotein (the major glycoprotein fraction of parotid saliva) contained 35% of the total saliva lipids and was enriched in phospholipids and cholesterol esters.

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Introduction.

Saliva is a complex mixture of proteins, glycoproteins, lipids, and inorganic ions.[1,2] A major constituent of parotid saliva is a low-molecular-weight (36,500), proline-rich, cationic glycoprotein.[3,4] This glycoprotein represents about 25% of the total parotid saliva protein and 75% of the carbohydrate.[1,3,4] The submandibular saliva contains all of the proteins found in parotid saliva, as well as the high-molecular-weight glycoproteins called mucins,[1] which are products of the mucous cells. The lipids of parotid and submandibular saliva consist primarily of neutral lipids and glycolipids, and of lesser quantities of phospholipids.[2,5-8]

Although salivary lipids have been implicated in salivary calculus and submandibular stone formation and mineralization,[7-10] the relationship of salivary lipids to the protein and glycoprotein components of saliva has not been extensively investigated.[11,12] Our purpose in the present study was to determine whether lipids show selectivity in their association with the protein and glycoprotein components in saliva.

Materials and methods.

Saliva collection. – Parotid and submandibular saliva (20 ml per individual) was collected from seven young adults having no clinical evidence of salivary gland pathology. The salivary flow was stimulated with 2% citric acid, and the first ml of saliva was discarded to avoid possible bacterial contamination.[7,8] The individual parotid and submandibular collections were pooled separately, were treated with buffered (pH 7.0) EDTA, and then filtered through 0.45 μm Millipore‡ filters.[7] The filtrates were dialyzed exhaustively against distilled water and were lyophilized.

Gel filtration. – A portion of each sample was dissolved in 6 M urea (50 mg of parotid saliva dissolved in 5 ml of urea and 62 mg of submandibular saliva dissolved in 5.5 ml of urea) and was applied separately to a column (2.0 x 150 cm) packed with Bio-Gel A-50 (50-100 mesh)¶ in 6 M urea. Elution was achieved with 6 M urea at a flow rate of 6.5 ml/h. Fractions of 4.8 ml were collected and monitored for carbohydrate content,[13] and optical density at 231 nm. The individual fractions were pooled as indicated in Figs. 1 and 2, were dialyzed against distilled water, and then lyophilized.

Lipid analysis. – The original samples of parotid and submandibular saliva, and the fractions obtained from the column were extracted with chloroform-methanol (2:1, v/v), and were filtered through grade F sintered glass funnels to retain the insoluble protein residue. The lipids contained in the extracts were dried, dissolved in a small volum of chloroform, and fractionated on salicic acid (100-200 mesh)¶ columns (0.5 x 15 cm) into neutral lipids, glycolipids, and phospholipids.[7] The neutral lipids, eluted from the columns with chloroform, were identified by cochromatography with authentic standards and were quantitated.[14] The glycolipids, eluted from the columns with acetone-methanol (8:2, v/v), were subjected to acid methanolysis, and their carbohydrate components were analyzed by gas chromatography.[7] The phospholipids, eluted from the columns with methanol, were quantitated according to the procedure of Lowry and Tinsley.[15]

Results.

Extraction of the dialyzed and lyophilized saliva with chloroform-methanol yielded 9.24 ± 0.83 mg of lipids/100 ml of parotid saliva and 9.52 ± 1.11 mg of lipids/100 ml of submandibular saliva. Of the total lipids in parotid saliva, about 77% was represented by the neutral lipids, 21% by the glycolipids, and 1.8% by the phospholipids. In submandibular saliva, the neutral lipids constituted 72% of the total lipids; glycolipids, 26%; and phospholipids, 2% (Table 1). The composition of the neutral lipid fraction of both types of saliva was similar: It consisted of free fatty acids, cholesterol, cholesterol esters, and mono-, di-, and triglycerides. In parotid saliva, the free fatty acids represented 53.3% of the neutral lipids; triglycerides, 21%; cholesterol

TABLE 1
LIPID COMPOSITION OF HUMAN PAROTID AND SUBMANDIBULAR SALIVA

Constituent (mg/100 ml of Saliva)	Parotid	Submandibular
Total Lipids	9.24 ± 0.83	9.52 ± 1.11
Free Fatty Acids	3.49 ± 0.35	3.12 ± 0.42
Mono- and Diglycerides	0.14 ± 0.04	0.15 ± 0.03
Triglycerides	1.37 ± 0.21	1.75 ± 0.30
Cholesterol	0.58 ± 0.13	0.58 ± 0.12
Cholesterol Esters	0.96 ± 0.20	0.89 ± 0.18
Glycolipids	1.78 ± 0.37	2.36 ± 0.34
Phospholipids	0.15 ± 0.02	0.18 ± 0.03

Each value represents means ± S.D. of triplicate analyses performed on the pool of saliva collected from seven individuals.
The dialyzed saliva yielded 210.5 mg of dry solid/100 ml of parotid saliva and 130.2 mg/100 ml of submandibular saliva.

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‡Millipore Corp., Bedford, MA
¶Bio-Rad, Richmond, CA

esters, 14.7%; cholesterol, 8.9%; and m no- and diglycerides, 2.1%. In submandibular saliva, the free fatty acids constituted 48.1% of the neutral lipids; triglycerides, 27%; cholesterol esters, 13.7%; cholesterol, 8.9%; and mono- and diglycerides, 2.3%.

Fractionation f the solubilized parotid saliva n a Bio-Gel A-50 column gave three peaks (Fig. 1). Two of the peaks consisted mainly of protein, while the most-included peak (Fraction III, Fig. 1), as judged by periodic acid/Schiff reaction, contained virtually all the carbohydrate and, hence, the proline-rich basic glycoprotein.[3,4] Gel filtration f the solubilized submandibular saliva also yielded three maj r peaks, two of which contained carbohydrate (Fig. 2). The excluded peak (Fraction I) contained about 35% of the carbohydrate – probably associated with acidic high-molecular-weight (Mr ≈ 2 x 10^6) mucous glycoprotein[1,16] – while peak III contained about 65% of the carbohydrate associated with the low-molecular-weight (Mr ≈ 2 – 2.5 x 10^6) glycoprotein.[16]

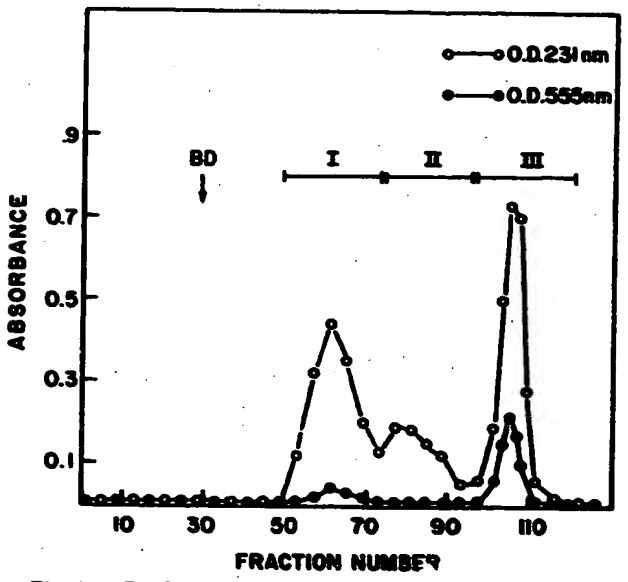

Fig. 1 – Gel filtration on Bio-Gel A-50 of parotid saliva. The sample (50 mg dissolved in 5 ml of 6 M urea) was applied to a column (2.0 x 150 cm) equilibrated with 6 M urea. Fractions of 4.8 ml were collected and were monitored for protein (absorbance at 231 nm, o-o-o) and carbohydrate (periodic acid/Schiff method, •-•-•). Tubes 49-73, 74-95, and 96-120 were pooled and designated as Fractions I, II, and III, respectively (BD = blue dextran).

Following dialysis and lyophilization, each fraction was extracted with chloroform-methanol, and their lipid content and composition were determined. The recovered parotid saliva fractions represented 79% of the dry weight of material applied to the column and 82% of its lipid. The corresponding recoveries for submandibular saliva were 71% and 70%, respectively. Of the total lipids recovered in parotid saliva fractions, 40.9% were in Fraction I, 24.1% in Fraction II, and 35% in Fraction III. In submandibular saliva, Fraction I contained 54.2% of the total lipids; Fraction II, 20.1%; and Fraction III, 25.7%. The percentage distribution of lipids in the fractions of parotid and submandibular saliva following gel filtration is given in Tables 2 and 3.

Although the various fractions of parotid and submandibular saliva exhibited similar lipid spectra, considerable differences were noted in their distribution. In parotid saliva (Table 2), Fraction I contained 40.9% of the total lipids, 48.1% of the total saliva-free fatty acids, and 47.5% of glycolipids; whereas the major glycoprotein-containing

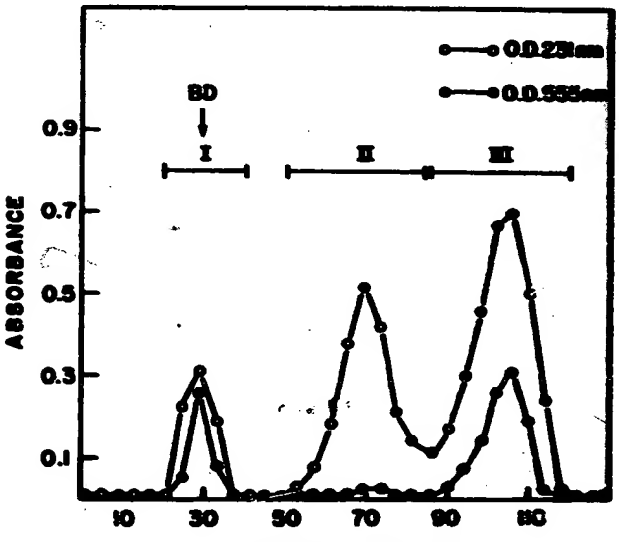

Fig. 2 – Gel filtration on Bio-Gel A-50 of submandibular saliva. The sample (62 mg dissolved in 5.5 ml of 6 M urea) was applied to a column (2.0 x 150 cm) equilibrated with 6 M urea. Fractions of 4.8 ml were collected and were monitored for protein (absorbance at 231 nm, o-o-o) and carbohydrate (periodic acid/Schiff method, •-•-•). Tubes 20-40, 50-84, and 85-120 were pooled and designated as Fractions I, II, and III, respectively (BD = blue dextran).

TABLE 2
DISTRIBUTION OF LIPIDS IN THE FRACTIONS OF PAROTID SALIVA FOLLOWING GEL FILTRATION ON BIO-GEL A-50

Constituent (μg/mg Dry Weight)	Fraction[a] I	II	III
Total Lipids	35.5 ± 6.2	52.8 ± 8.5	46.5 ± 5.7
Free Fatty Acids	16.7 ± 2.4	21.1 ± 2.2	13.8 ± 1.9
Triglycerides	3.1 ± 0.3	11.8 ± 1.3	8.3 ± 0.9
Cholesterol	1.8 ± 0.2	3.7 ± 0.4	3.4 ± 0.2
Cholesterol Esters	3.4 ± 0.3	3.5 ± 0.5	6.9 ± 0.8
Glycolipids	8.5 ± 0.9	7.9 ± 0.6	9.0 ± 0.6
Phosph lipids	0.5 ± 0.0	0.6 ± 0.1	1.0 ± 0.2

Each value represents means ± S.D. f triplicate analyses.
[a]Fraction I represented 47.7% f the dry weight of recovered material; Fraction II, 19.3%; and Fraction III, 33.0%.

TABLE 3
DISTRIBUTION OF LIPIDS IN THE FRACTIONS OF SUBMANDIBULAR SALIVA FOLLOWING GEL FILTRATION ON BIO-GEL A-50

Constituent (μg/mg Dry Weight)	Fraction[a] I	II	III
Total Lipids	178.4 ± 21.3	41.1 ± 5.0	44.6 ± 5.3
Free Fatty Acids	69.1 ± 9.8	7.9 ± 0.9	13.8 ± 2.1
Triglycerides	14.9 ± 1.2	16.2 ± 1.1	10.2 ± 0.9
Cholesterol	11.2 ± 1.1	2.9 ± 0.3	2.1 ± 0.2
Cholesterol Esters	13.1 ± 1.4	4.3 ± 0.3	5.5 ± 0.6
Glycolipids	55.2 ± 5.1	6.1 ± 0.4	8.7 ± 0.5
Phospholipids	3.2 ± 0.4	0.5 ± 0.1	1.1 ± 0.2

Each value represents means ± S.D. of triplicate analyses.
[a]Fraction I represented 22.2% of the dry weight of recovered material; Fraction II, 35.7%; and Fraction III, 42.1%.

fraction (Fracti n III) contained 35% of the t tal lipids, 49.6% of the cholesterol esters, 47.1% of phospholipids, 41.9% f ch lesterol, and 41.8% of triglycerides. In submandibular saliva (Table 3), Fracti n I contained 54.2% f the total lipids, 67.6% f the total saliva glycolipids, 63.9% of free fatty acids, 56.3% f ch lesterol, and 42.9% f ch lesterol esters. The content f triglycerides, however, was higher in Fraction II (43.3% of the total), even though it contained only 20.1% of the total lipids.

Discussion.

The organic components of alimentary tract secretions, including saliva, consist of a variety of proteins, glycoproteins, and lipids.[1-8,14] In the oral environment, some of these substances interact with cariogenic microorganisms and, thus, play a major role in the defense against cariogenic challenges.[17] The protein and glycoprotein components of saliva also interact with the inorganic constituents of saliva and become selectively incorporated to the tooth surfaces in a form of pellicle which serves as a diffusion barrier, as well as a focal point in attachment and color ation of the tooth surfaces by the cariogenic bacteria.[18]

Among the salivary components capable of affecting the interactions of salivary proteins and glycoproteins with inorganic ions are lipids.[11,12] Although direct evidence of the involvement of salivary lipids in the defense against cariogenic microorganisms is not available, several studies indicate that lipids affect the viscosity and solubility of the secretions, facilitate penetration of the mucosa by lipophilic substances, and alter the association of calcium with salivary glycoproteins.[11,12,19,20] Some of the salivary lipids are also capable of enhancement of the glucosyltransferase activity associated with the cariogenic potential f the bacteria.[21]

These diverse effects are potentially significant in a variety of processes occurring in the oral cavity, including the successful defense of the eeth against bacterial challenge.[22] Yet, the relationship of salivary lipids to the protein and glycoprotein components of saliva, and the extent of their interaction, is not well understood.

The results presented in this study show that lipids in parotid and submandibular saliva exhibit quite specific association with the protein and glycoprotein components. Although each of the fractions of saliva separated by gel filtration exhibits a similar lipid spectrum, the percentage distribution of total lipids, as well as the distribution of individual lipids among the fractions, differs considerably. In parotid saliva, the free fatty acids are found mainly in th fraction virtually devoid of carbohydrate, while most of the cholesterol esters and phospholipids are associated with th carbohydrate fraction. In submandibular saliva, the majority of lipids and, in particular, glycolipids and free fatty acids are associated with the fraction containing the high-molecular-weight mucous glycoproteins.

The observed distribution of lipids among the protein and glycoprotein components of saliva is quite similar to that reported for the lipids of gastric mucous,[23] where lipids and glycolipids, in particular, are found in the gastric mucin fraction. The specific nature of the interaction of lipids with proteins and glycoproteins in saliva and possible effects n the physico-chemical or bi l gical properties f these substances remain t be established.

Conclusions.

The lipid content and compositi n f human parotid and submandibular saliva were determined, and the distributi n of vari us lipids among the fracti ns separated by gel filtrati n n Bio-Gel A-50 was investigated. Both types f saliva, while exhibiting a similar total lipid content and spectrum, sh wed considerable differences in the distributi n f individual lipid components among the separated fractions. The free fatty acids in parotid saliva were associated (72%) with the carbohydrate-poor fractions, while the carbohydrate-rich fraction was enriched in phospholipids and cholesterol esters. In submandibular saliva, the majority of lipids and, in particular, glycolipids and free fatty acids was associated with the fraction containing high-molecular-weight mucous glycoproteins. The results indicate that, in saliva, lipids are associated with protein and glycoprotein components.

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CALL FOR ABSTRACTS

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The Dental Health Section of the American Public Health Association is soliciting papers addressing a broad range of dental public health subjects to be submitted for presentation at the Association's 111th Annual Meeting, to be held in Dallas, Texas, on November 13-17, 1983. Specific invitation is extended to papers which complement the meeting's theme: "Science and Social Action – Promoting Health and Peace." Examples of possible subject areas are: promoting oral health, prevention of communicable disease transmission in dental public health programs, alternative dental care delivery models, program management and evaluation, oral health for special population groups – adolescents, handicapped, and elderly – occupational health, use and abuse of radiographs, cost containment, and related topics.

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A typed outline of the paper, at least one page long, must be included with the abstract to assist in the review and selection process. If an abstract is selected, the complete paper is expected by October 4, 1982.

The deadline for submission of abstracts is March 11, 1983. An abstract form can be found in the January, 1983, issue of *The Nation's Health.* Abstract forms and additional information can be obtained by writing to:

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(FILE 'HOME' ENTERED AT 13:58:18 ON 25 JUN 2001)

FILE 'MEDLINE, EMBASE, SCISEARCH, USPATFULL, CAPLUS' ENTERED AT 13:59:18
ON 25 JUN 2001

L1	6312	S (APOLIPOPROTEIN? AND LIPOPROTEIN?) AND ?ASSAY?
L2	348	S ((APO!A? OR APOA?) AND (APO!B? OR APOB?)) AND ?ASSAY?
L3	279	S L1 AND L2
L4	12	S L3 AND ((DRY CHEMI?) OR ?STRIP? OR DIPSTICK?)
L5	12	DUP REM L4 (0 DUPLICATES REMOVED)
L6	39	S L1 AND SALIVA
L7	1	S L6 AND L2
L8	39	DUP REM L6 (0 DUPLICATES REMOVED)
L9	2	S L2 AND SALIVA?
L10	15	S L2 AND ((DRY CHEMI?) OR ?STRIP? OR DIPSTICK?)
L11	15	DUP REM L10 (0 DUPLICATES REMOVED)
L12	1	S L11 AND SALIVA?
L13	349	S (APOLIPOPROTEIN? OR LIPOPROTEIN?) (6P) (SALIVA?)
L14	2	S ((APO!A? OR APOA?) AND (APO!B? OR APOB?)) AND SALIVA?

L5 ANSWER 12 OF 12 USPATFULL

AB A method is provided, in one embodiment, for the determination of an analyte in a biological fluid sample in the presence of a substance interfering with an **assay** for the analyte. This embodiment is implemented by using antibodies to cause the selective immunoreaction of at least one of the analyte or the interfering substance and then conducting an **assay** for the analyte in at least one of the immunoreactants or the non-reactants. Another embodiment provides a disposable reaction device to implement the method. The invention is applicable to the detection of a wide variety of analytes, including cholesterol in a targeted **lipoprotein** class in the presence of cholesterol in another class; to targeted isozymes of enzymes such as creatine kinase, lactate dehydrogenase, amylase, and alkaline or acid phosphatases in the presence of other isozymes; as well as to targeted immunoglobulins in the presence of non-targeted immunoglobulins.

ACCESSION NUMBER: 95:29547 USPATFULL

TITLE: Determination of analytes in biological fluids in the presence of substances interfering with **assays** therefor

INVENTOR(S): Ollington, James F., Chelmsford, MA, United States
Byrnes, Ronald J., West Bridgewater, MA, United States
Pogorzelski, Donald E., Nashua, NH, United States

PATENT ASSIGNEE(S): Genzyme Corporation, Cambridge, MA, United States (U.S. corporation)

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PRIMARY EXAMINER: Scheiner, Toni R.
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LEGAL REPRESENTATIVE: Bromberg & Sunstein
NUMBER OF CLAIMS: 16
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 20 Drawing Figure(s); 13 Drawing Page(s)
LINE COUNT: 924
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

TI Determination of analytes in biological fluids in the presence of substances interfering with **assays** therefor

AB . . . for the determination of an analyte in a biological fluid sample in the presence of a substance interfering with an **assay** for the analyte. This embodiment is implemented by using antibodies to cause the selective immunoreaction of at least one of the analyte or the interfering substance and then conducting an **assay** for the analyte in at least one of the immunoreactants or the non-reactants. Another embodiment provides a disposable reaction device. . . . the method. The invention is applicable to the detection of a wide variety of analytes, including cholesterol in a targeted **lipoprotein** class in the presence of cholesterol in another class; to targeted isozymes of enzymes such as creatine kinase, lactate dehydrogenase,. . .

SUMM The present invention relates to the determination of analytes in the presence of substances interfering with **assays** for such analytes. The invention also relates to the use of devices for implementing such determination. The invention can be used for the

detection, in a biological fluid sample, of analytes in targeted **lipoprotein** classes; and targeted isozymes of creatine kinase, lactate dehydrogenase, amylase, and alkaline or acid phosphatases; and targeted immunoglobulins; as well. . .

SUMM . . . A., eds. The C. V. Mosby Company, St. Louis, Mich.); pp. 454-483 (1989)). Similarly, the detection of cholesterol, in specific **lipoprotein** classes, is used in the determination of coronary heart disease risk. (Russel et al. "Lipids" in Clinical Chemistry (Kaplan, L.. . .

SUMM The efficacy of **assays** for analytes in a biological fluid sample can be reduced due to the presence of substances which interfere with the **assay** (Kaplan, L. and Pesce, A., "Interferences in Chemical Analysis" in Clinical Chemistry (Kaplan, L. and Pesce, A., eds., The C.. . . (1989)). For example, compounds such as hemoglobin or bilirubin, which have a strong visible absorbance, can interfere with a spectrophotometric **assay** for an analyte. Kaplan and Pesce, id.

SUMM . . . therefore traceable to the National Reference System for Cholesterol. Due to the absence of accepted National Reference Systems for triglycerides, **lipoproteins**, and **apolipoproteins**, much remains to be done in the elimination of interlaboratory variability associated with these lipid related tests. Presently, these tests. . .

SUMM . . . of the art in the detection of analytes in biological fluids. The prior art known to the inventors lacks an **assay**, for cholesterol in specific **lipoprotein** classes, that is simultaneously (i) easily interpretable from an epidemiological point of view; (ii) easily, quickly and inexpensively implemented, and (iii) universally applicable to all routine clinical chemistry testing systems. Indeed, the inventors are unaware of any **assays**, for specific classes of an analyte that are the subject of the routine tests described above, that meets these two. . .

SUMM Triglycerides and cholesterol are transported in the blood via **lipoprotein** particles. Abnormalities in these **lipoproteins**, either inherited, environmentally contributed, or a combination of both, lead to a variety of disorders including a predisposition to premature. . .

SUMM The chemistry, biosynthesis, function, metabolism, cell biology, and molecular genetics of **lipoprotein** particles have been extensively reviewed (Segrest, Jere P., and Albers, John J., editors, 128 Methods in Enzymology (1986) and Albers,. . .

SUMM **Lipoprotein** particles are divided into four major classes based on their density, composition, and electrophoretic mobility: The classes are chylomicrons, very low density **lipoproteins** (VLDL), low density **lipoproteins** (LDL) and high density **lipoproteins** (HDL). LDL and HDL particles may be further subdivided on the basis of density. The **lipoprotein** particles are composed of triglycerides, cholesterol, fatty acids esters of cholesterol, phospholipid and protein. The varying ratios of protein to lipid, in different **lipoprotein** classes, account for the physical differences by which these particles can be fractionated by density gradient centrifugation.

SUMM The protein components, known as **apolipoproteins**, are responsible for a variety of cellular functions. Increased levels of LDL cholesterol and decreased levels of HDL cholesterol have been shown to be risk factors for CHD. Consequently, clinical diagnostic **assays** for cholesterol content in the major **lipoprotein** classes are performed extensively and a large body of statistical data on the normal ranges for these classes is available. . .

SUMM In the clinical laboratory, the following **assays** are performed routinely to characterize the lipid and cholesterol profile of a plasma or serum sample: (i) Triglycerides are determined. . .

SUMM . . . by phosphotungstate, plus divalent cations, with subsequent total cholesterol measurement of the separated supernatant. In general, cholesterol detection in specific **lipoprotein** classes lacks a standardized reference system.

SUMM More recent efforts toward separation and quantitation of **lipoprotein** classes have utilized antibodies, either polyclonal or monoclonal, directed against **apolipoproteins** which are specific to distinct, clinically relevant **lipoprotein** particles (Tikkanen et. al., J. Lipid Research 24:1494-1498 (1983); and Ordovas et. al. J. Lipid Research 28:1216-1224 (1987)).

SUMM In research laboratories a variety of immuno-based analytical techniques have been employed to quantitate **lipoproteins**, including radial immunodiffusion, **radioimmunoassay** and **electroimmunoassay**, but these techniques are too cumbersome to be employed in a clinical diagnostic setting where large numbers of samples must be handled rapidly. This disadvantage may be addressed by using an enzyme-linked immunoabsorbant **assay** (ELISA), and this is an area of active investigation (Ordovas et. al., J. Lipid Research 28:1216-1224 (1987)).

SUMM . . . some of these immuno-based techniques, including ELISA suffer, and that is that these procedures quantitate an epitope associated with specific **lipoprotein** classes--they do not measure cholesterol levels. The significance of this situation is that there must be a very large number. . .

SUMM . . . known. Longenecker (U.S. Pat. No. 4,302,536 (1981)) reported the determination of antigenic materials in biological fluids and cells by colorimetric **immunoassay** with an adduct of antibody and chromo-protein. Onishi and Ito (Eur. Pat. No. 327,918 (1989)) reported an **immunoassay** using the homogeneous competitive reaction between a target and labelled substance and a specific binder. Freytag and Ishikawa (U.S. Pat. No. 4,657,853 (1987)) reported a high sensitivity **immunoassay** using a polymeric enzyme-antibody conjugate. Nippon (Jap. Pat. No. 59226864 (1984)) reported an **immunoassay** in which levels of transforming growth factor (TGF) in a liquid sample are detected using an immobilized TGF antibody and an enzyme labelled TGF antibody. Gomez and Wicks (U.S. Pat. No. 4,353,982 (1982)) report an **immunoassay** for creatine kinase in blood serum using iodine-125 labelled antibody to precipitate immune complex mixtures.

SUMM Several groups have examined selective immunoprecipitation of specific **lipoprotein** classes followed by cholesterol quantitation in the **lipoprotein** class remaining in solution. Heuck et al. reported the use of antibodies to **ApoB** to precipitate LDL and VLDL followed by measuring cholesterol levels in the HDL left in the supernatant. Antibodies to **apoAI** and apoC were also used, to precipitate HDL and VLDL, followed by determination of cholesterol levels in the LDL left. . . Germany Patent No. P32 15 310 (1983); Kerscher et al. Clin. Bioch. 18:118-125 (1985)). Antibodies to both apoproteins and whole **lipoproteins**, including immobilized antibodies, have been used to immunoprecipitate **lipoproteins** followed by determination of the cholesterol content of the **lipoprotein** class remaining in solution (Ziegenhorn et al. Canadian Patent No. 1 211 707 (1986)). This reference, however, does not describe. . .

SUMM . . . a method for detecting an analyte in a biological fluid sample in the presence of a substance interfering with an **assay** for the analyte. This embodiment is implemented by using antibodies to cause the selective immunoprecipitation of an interfering substance and then conducting an **assay** for the analyte the non-reactants. In another embodiment, the invention provides a disposable reaction device to implement the method.

SUMM . . . applicable to the detection of a wide variety of biological

analytes, including but not limited to cholesterol in a targeted **lipoprotein** class in the presence of cholesterol in another class; as well as to targeted isozymes of creatine kinase, lactate dehydrogenase,. . .

DRWD FIG. 1 is a schematic illustration of the sequential density gradient ultracentrifugation separation of the **lipoproteins** used as antigens for obtaining antisera used in Example 1.

DRWD FIG. 4 is a graph showing the time course for the precipitation of human plasma **ApoB**-containing **lipoprotein** particles by freeze-dried anti-ND-LDL sera as determined by supernatant cholesterol estimation, in accordance with Example 1.

DRWD FIG. 6 is a graph supernatant cholesterol and **ApoB** levels in accordance with Example 1.

DETD . . . for the detection of an analyte in a biological fluid sample, in the presence of a substance interfering with an **assay** for such analyte, using immunoseparation technology. In the invention, antibodies are used to cause selective immunoreaction of a substance interfering with an **assay** for the analyte, and then the analyte is detected by **assay** of the non-reactants. In one embodiment of the invention, a reaction device is used to rapidly and inexpensively implement the. . . linked dextran, cross linked polysaccharides, or cross linked acrylamide, microporous filters or membranes. Other suitable insoluble carriers include a coiled **strip** or the interior wall of the reaction device itself. Antibodies may also be bound to soluble large MW polymers to. . .

DETD . . . the prior art. Embodiments of the invention may be used in connection with the determination of cholesterol in an immunoseparated **lipoprotein** class based on conventional enzymatic techniques. Because the invention permits utilization of existing routine clinical tests (applied to the immuno-separated **lipoprotein** class), the results of testing, in accordance with a preferred embodiment of the invention, may be directly related to the national reference system. (Other immuno-based **assays** currently provided by embodiments of the invention include apoliproprotein quantitation, for which statistically significant clinical data, i.e., normal ranges in. . . example, a plasma or serum sample may be first analyzed for total cholesterol in the absence of any separation of **lipoprotein** fractions. Subsequently, aliquots of the original sample may be subjected to immunoprecipitation by various specific antibody preparations either sequentially or. . . and comparing this sum to the unfractionated value. The use of antibodies in the invention permits highly specific fractionation of **lipoprotein** classes.

DETD . . . for characteristics relevant to other immuno-based techniques, e.g., good binding to plastic surfaces as needed for ELISA procedures. In addition, **assay** in accordance with this invention may be implemented with a device containing stabilized antibodies, which allows rapid, inexpensive, and efficient. . .

DETD The embodiments described below are discussed principally in the context of the detection of cholesterol in specific **lipoprotein** classes. However, as shown below, the invention is equally applicable to the detection of a wide variety of other analytes.

DETD 1. Narrow density **lipoprotein** fractions

DETD Pooled human plasma in CPD, collected at the New England Medical Center Blood Bank, was used for the preparation of **lipoprotein** fractions for use as antigens. The plasma, at an assumed density of 1.006 (g/ml), was fractionated by sequential ultracentrifugation as. . .

DETD . . . C., 45 k rpm in a Ti60 rotor. Following centrifugation the tubes were stored on ice and the 1.006 floating **lipoprotein** fraction was sliced off the top of the tube, cutting close to the lower interface. The 1.006 bottom fractions were. . .

DETD . . . under the same conditions as above and the collected tubes were
maintained on ice prior to slicing off the top **lipoprotein** layer. This top layer was sliced just at the lower interface; the 1.05 top layers were pooled and contain the. . .

DETD . . . 50 k rpm, 42-48 hr in a Ti60 rotor. The collected tubes were maintained as described above and the upper **lipoprotein** fraction was sliced off close to the lower interface. The 1.107 bottom fractions were pooled, filtered and the density adjusted. . . interface of the top layer to yield a 1.19 top fraction containing narrow density HDL (ND-HDL). The fractionation of the **lipoprotein** particles through sequential ultracentrifugation is summarized schematically in FIG. 1. The harvested narrow density fractions were washed and refractioned by. . .

DETD . . . fraction was determined by the standard methods of either Lowry
or using the Biorad reagents. The preparation of narrow density **lipoprotein** fractions described above is an improvement on the method described by Schumaker, V. N. and Poppione, O. L. (1986) Methods.
. .

DETD The yields of narrow density **lipoprotein** fractions isolated in this manner are typically: from 266 ml plasma, 31 ml of ND-LDL at 1.2 g/ml protein and. . .

DETD Starting with purified ND-LDL, **ApoB** was isolated by electrophoresis through a preparative 15% polyacrylamide gel followed by
excision of the separated **ApoB** band. Upon completion of electrophoresis the gel was stained with sodium acetate to visualize protein bands, as described in E. Harlow and D. Lane, editors, Antibodies. A Laboratory Manual (Cold Spring Harbor Press, 1988). The **ApoB** containing gel region was excised using a scalpel or razor and the polyacrylamide gel was homogenized by repeated passages though progressively narrower gauge needles according to the method described essentially in Antibodies, ibid. The **ApoB** in homogenized acrylamide may be stored at @ 4.degree. C. prior to immunization.

DETD Starting with approximately 10 ml purified ND-HDL, **ApoAI** and **ApoAII** were purified by chromatography over Sephacryl S-200 essentially as described by Brewer, et al. (1986), Methods in Enzymology
128:223-246. Fractions containing separated **ApoAI** and AII were quantitated for protein by Biorad **assay** and electrophoresed through a preparative 15% polyacrylamide gel under denaturing conditions. Protein bands were visualized, excised and prepared for immunization. . .

DETD It is within the scope of the invention to use purified **ApoAI** and **ApoAII** to immunize individual goats, and obtain the corresponding antisera.

DETD . . . be free of material cross-reactive to any of the apoproteins of
purified HDL. The ND-LDL antisera showed cross-reactivity only to **ApoB** in VLDL, LDL, and whole plasma.

DETD The anti-narrow density HDL sera showed reactivity to **ApoAI** in HDL and whole plasma and reactivity to ApoCs and ApoE in HDL, VLDL and whole plasma. A small amount of cross-reactive material to **ApoB** was detected and can be removed by immunoaffinity chromatography over an
ND-HDL-sepharose column. The column is prepared in the following. . .

DETD Antisera was characterized further by examining immunoreactivity against
lipoprotein particles that had been electrophoresed through an agarose gel under non-denaturing conditions using the Corning Agarose Universal Electrophoresis System.RTM.. Following. . .

DETD The anti-ND-LDL sera showed cross-reactivity with the Beta migratory **lipoprotein** region only; no reactivity was detectable either to the alpha migrating region or to albumin.

DETD ND-LDL antisera purification was monitored by Western blot by the following procedure. The **lipoprotein** particles were electrophoresed through an SDS-PAG described above followed by electrotransfer to nitrocellulose (S & S) using the following conditions:. . .

DETD Immunoprecipitation of Beta **lipoproteins** in human plasma using anti-ND-LDL sera was examined and compared with the prior art. 200 .mu.l of fresh human plasma. . .

DETD The filtrate was **assayed** for total cholesterol using an Abbott ACA 200 chemistry analyzer and Abbott A-Gent cholesterol reagents, although any cholesterol determination method. . . for 19 human plasma samples compared with values obtained using dextran sulfate (50 kd molecular weight) as a control Beta **lipoprotein** precipitating agent, used essentially as described by Warnick, et. al (1985). FIG. 3 indicates the between run precision of the. . .

DETD Apoprotein **immunoassays** were used to evaluate the comparative specificity of **lipoprotein** particle separation between the immunoseparation method and the dextran sulfate method. FIG. 6 demonstrates the immunoprecipitation of **lipoprotein** particles by anti-ND-LDL sera monitored by the reduction in supernatant (filtrate) cholesterol values (as described above) and by Apoprotein B **immunoassay** measured by the method of Ordovas, et al. (1987). At the equivalent of 350 .mu.l anti-ND-LDL sera, the supernatant cholesterol value was 32.9 mg/dl, the dextran sulfate control value was 30.0 mg/dl. By **immunoassay**, the following results were obtained: at 350 .mu.l of antisera the supernatant **ApoB** level was undetectable above the background control; the dextran sulfate supernatant yielded 1.2 g/dl **ApoB** remaining in the supernatant.

DETD . . . its base, and (iii) an immobilized antibody component 76 which may, for example, be antibody-coated, or beads or a coiled **strip**, such as represented in FIG. 8), which is contained within the reaction chamber 74. Any other suitable means for immobilization. . .

DETD . . . a screw cap gripping the threaded shoulder 73 in FIG. 10) or the filtrate or an aliquot thereof may be **assayed** for non-reactant analyte(s), using routine **assays**.

DETD . . . at its bottom. The reaction pipette 114 contains prepacked aliquots of antibodies immobilized, for example, on beads or a coiled **strip** (FIG. 13), or otherwise in the manner as discussed above in connection with FIG. 8. The reaction pipette 114 may. . .

DETD . . . the operation of the embodiment described above in connection with FIGS. 11-17. The collected filtrate or immunoreactants can then be **assayed** using routine tests.

DETD Although the above discussion has been with respect primarily to the detection of cholesterol in specific **lipoprotein** classes, the invention is also widely applicable to the detection of a targeted analyte in a class of analytes, such. . .

DETD . . . routine test for the class of analytes, then following separation in accordance with the invention, the targeted analyte can be **assayed** using the routine test for the class of analytes. For example, one may use a routine test for amylase to. . . separation, for example, using antibodies to all isozymes of amylase other than pancreatic specific amylase. Thereafter the filtrate may be **assayed** using the routine test to identify the level of pancreatic specific amylase in the sample. A similar strategy may be used to **assay** any targeted analyte in a class of analytes for which a routine test exists.

DETD . . . invention may also be used to remove substances such as bilirubin and hemoglobin that can interfere with spectrophotometric or other **assays** for an analyte. In such instances, antibodies to bilirubin and hemoglobin may be employed to achieve their immunoseparation (using the invention) from the sample prior to conduct

of an **assay** in accordance with prior art techniques.

CLM What is claimed is:

1. A method for measuring a cholesterol analyte in a targeted **lipoprotein** class in a biological fluid in the presence of at least one cholesterol-containing interfering substance in another **lipoprotein** class, comprising: (i) obtaining a disposable reaction device having a reaction chamber, the reaction chamber containing immobilized antibodies which are. . . chamber, wherein the

reacted interfering substance remains in the reaction chamber of the reaction device; and (iv) conducting a clinical **assay** for the cholesterol analyte on the non-reactants including the cholesterol analyte.

15. A method for measuring a cholesterol analyte in a targeted **lipoprotein** class in a biological fluid in the presence of at least one cholesterol-containing interfering substrate in another **lipoprotein** class, comprising: (i) selecting a disposable reaction device having a reaction chamber; (ii) immobilizing antibodies which are specific for said. . . analyte; (iv) separating the non-reactants from step (iii), including the cholesterol, analyte from the interfering substance; and (v) conducting an **assay** for the cholesterol analyte on the non-reactants including the cholesterol analyte.

16. A method for measuring a cholesterol analyte in a targeted **lipoprotein** class in a biological fluid in the presence of at least one cholesterol-containing interfering substance in another **lipoprotein** class, comprising: (i) obtaining a disposable reaction device having a reaction chamber, the reaction chamber containing antibodies which are specific. . . the cholesterol analyte; (iii) separating non-reactants including the cholesterol analyte from bound interfering substance by filtration; and (iv) conducting an **assay** for the cholesterol analyte on the filtrate, wherein the filtrate includes the cholesterol analyte.

L7 ANSWER 1 OF 1 USPATFULL

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TITLE:	Chromosome 11-linked coronary heart disease susceptibility gene CHD1
INVENTOR(S):	Ballinger, Dennis G., Menlo Park, CA, United States Ding, Wei, Salt Lake City, UT, United States Wagner, Susanne, Murray, UT, United States Hess, Mark A., Salt Lake City, UT, United States
PATENT ASSIGNEE(S):	Myriad Genetics, Inc., Salk Lake City, UT, United States (U.S. corporation)

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ASSISTANT EXAMINER:	Chakrabarti, Arun
LEGAL REPRESENTATIVE:	Fish & Neave, Haley, Jr., Esq., James F., Liang, Stanley D.
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EXEMPLARY CLAIM:	1
NUMBER OF DRAWINGS:	12 Drawing Figure(s); 12 Drawing Page(s)
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CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB Human coronary heart disease susceptibility gene (CHD1), some alleles of which are related to susceptibility to coronary heart disease. Germline mutations in the CHD1 gene and their use in the diagnosis of predisposition to coronary heart disease and to metabolic disorders, including hypoalphalipoproteinemia, familial combined hyperlipidemia, insulin resistant syndrome X or multiple metabolic disorder, obesity, diabetes and dyslipidemic hypertension. Presymptomatic therapy of individuals who carry deleterious alleles of the CHD1 gene (including gene therapy, protein replacement therapy, and administration of protein mimetics and inhibitors). The screening of drugs for dyslipidemic therapy.

SUMM Also within the scope of this invention are binding **assays** utilizing the proteins of the invention.

SUMM Some risk factors appear relatively simple genetically. For instance, **lipoprotein** (a) (Lp(a)) levels are strongly correlated with CHD. Greater than 95% of the variation in Lp(a) protein levels is associated.

. . . The familial hypercholesterolemia (FH) syndrome is a rare syndrome (affecting about 1 in 500 individuals) characterized by very high low-density **lipoprotein** (LDL)-cholesterol, and very early CHD, usually manifest in the 20s or 30s. Early family studies identified and clinically defined obligate. . .

SUMM . . . the other hand, some dyslipidemias appear to be genetically quite complex. For instance, about half of the variation in high-density **lipoprotein**-C (HDL-C) levels appear to be genetically determined (Friedlander, et al., 1986a; Friedlander, et al., 1986b; Moll, et al.,

1989; Perusse,. . . 1989; Prenger, et al., 1992; Cohen, et al., 1994). Defects in several genes are known to cause low HDL-C including **apolipoprotein** AI (**ApoAI**) deficiency, **apolipoprotein** B (**ApoB**) polymorphisms (Peacock et al., 1992), **lipoprotein** lipase (LPL) deficiency and lecithin:cholesterol acetyltransferase (LCAT) deficiency (recently reviewed in Funke, 1997). However, in aggregate these known genetic defects. . . proportion of individuals with low HDL. Some studies have shown association of HDL-C levels with the hepatic triglyceride lipase and **ApoAI**, CIII AIV loci (e.g. Cohen, et al., 1994), indicating that a significant portion of the genetic effects may come from. . . failed to find such an association (Bu, et al., 1994; Maheny, et al., 1995; Marcil, et al., 1996). Additionally, the **ApoAI**, CIII and AIV loci have been associated with familial combined hyperlipidimia (FCH) in some studies (Wojciechowski, et al., 1991; Tybjaerg-Hansen,. . .

SUMM Also within the scope of this invention are methods to screen drugs (e.g. binding **assays**) for inhibition or restoration of CHD1 gene product function as a therapy for coronary heart disease or metabolic disorders.

DRWD . . . 3 through 8). No d(I:C) competitor was used. Relative to the start of transcription, the probes spanned: -573 to -165 (**apolipoprotein** AIV), -743 to -366 (**apolipoprotein** CIII, Kardassis, et al, 1996), -532 to -187 (**Lipoprotein** Lipase). The molar protein:probe ratio is indicated above each lane. 100.times. protein corresponds to approximately 140 nM in the binding. . .

DRWD FIG. 5B. Mobility shift of **Apolipoprotein** AIV gene promoter subfragments by CHD1.ZnF3-8 Protein. The promoter fragment (-573 to -165) shifted by CHD1 GST fusion protein was. . .

DRWD . . . 8). Poly d(I:C) competitor was used as indicated. Relative to the start of transcription, the probes spanned: -573 to -165 (**Apolipoprotein** AIV, Apo AIV), -1304 to -968 (Lecithin:cholesterol acetyltransferase, LCAT), -324 to +16 (**Apolipoprotein** E, Apo E). The molar protein:probe ratio in the binding reaction was 100.times. (GST, 250.times.); protein concentration was approximately 140. . .

DRWD FIG. 6. Diagrammatic summary of gel shift **assay** results for fragments of the **Apolipoprotein** AIV promoter (FIG. 6A), the **Apolipoprotein** CIII enhancer (FIG. 6B) and the **lipoprotein** lipase (LPL) promoter (FIG. 6C). Fragments marked with "B" bind to CHD1.ZnF3-8 as detected by a probe mobility shift on. . .

DRWD FIG. 7. The regulatory regions of the **ApoAIV** gene, the ApoCIII enhancer, and the ApoE gene, and fragments that bind CHD1.ZnF.3-8. Diagrams of promoter fragments from these genes,. . .

DETD . . . for specific immunoreactivity with CHD1 polypeptide or fragments thereof. See, Harlow and Lane, 1988. These antibodies will be useful in **assays** and as pharmaceuticals.

DETD An immunological response is usually **assayed** with an **immunoassay**. Normally, such **immunoassays** involve some purification of a source of antigen, for example, that produced by the same cells and in the same fashion as the antigen. A variety of **immunoassay** methods are well known in the art. See, e.g., Harlow and Lane, 1988, or Goding, 1986.

DETD . . . to, e.g., plasma, serum, spinal fluid, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, **saliva**, blood cells, tumors, organs, tissue and samples of in vitro cell culture constituents.

DETD . . . which are detected by amplification of the target nucleic acid sequence using PCR; such markers are highly informative, easy to **assay** (Weber and May, 1989), and can be **assayed** simultaneously using multiplexing strategies (Skolnick and Wallace, 1988), greatly reducing the number of experiments required. This linkage

analysis is described. . .

DETD . . . fluorescent in situ hybridization (FISH), direct DNA sequencing, PFGE analysis, Southern blot analysis, single stranded conformation analysis (SSCA), RNase protection **assay**, allele-specific oligonucleotide (ASO) analysis, dot blot analysis and PCR-SSCP, as discussed in detail further below. Also useful is the recently. . .

DETD . . . optimal conditions, mutations in the coding sequence of a gene are rarely missed. Another approach is the single-stranded conformation polymorphism **assay** (SSCA) (Orita et al., 1989). This method does not detect all sequence changes, especially if the DNA fragment size is. . . transcription or translation of the protein. Other methods that might detect these classes of mutations such as a protein truncation **assay** or the asymmetric **assay**, detect only specific types of mutations and would not detect missense mutations. A review of currently available methods of detecting. . .

DETD 3) RNase protection **assays** (Finkelstein et al., 1990; Kinszler et al., 1991);

DETD 7) single nucleotide extension **assays**.

DETD . . . used, as disclosed in European Patent Application Publication No. 0332435 and in Newton et al., 1989. For single nucleotide extension **assays**, primers are used with their 3' ends at the nucleotide prior to a particular CHD1 mutation. The primers are then. . . to the oligonucleotide. The genotype of the individual can be inferred from the pattern of nucleotides added in the extension **assay**. Insertions and deletions of genes can also be detected by cloning, sequencing and amplification. In addition, restriction fragment length polymorphism. . .

DETD In the first three methods (SSCA, DGGE or CDGE and RNase protection **assay**), a new electrophoretic band appears. SSCA detects a band that migrates differentially because the sequence change causes a difference in. . . differences in migration rates of mutant sequences compared to wild-type sequences, using a denaturing gradient gel. In an allele-specific oligonucleotide **assay**, an oligonucleotide is designed that detects a specific sequence, and the **assay** is performed by detecting the presence or absence of a hybridization signal. In the mutS **assay**, the protein binds only to sequences that contain a nucleotide mismatch in a heteroduplex between mutant and wild-type sequences.

DETD . . . mutation. Antibodies specific for products of mutant alleles could also be used to detect mutant CHD1 gene product. Such immunological **assays** can be done in any convenient formats known in the art. These include Western blots, immunohistochemical **assays** and ELISA **assays**. Any means for detecting an altered CHD1 protein can be used to detect alteration of wild-type CHD1 genes. Functional **assays**, such as protein binding determinations, can be used. In addition, **assays** can be used that detect CHD1 biochemical function, for instance, DNA binding. Finding a mutant CHD1 gene product indicates presence. . .

DETD The genes to whose promoters CHD1 binds can be grouped according to function. The first class is a set of **apolipoprotein** genes that encode structural components of circulating **lipoproteins**. The second class is a set of genes encoding enzymes known to influence **lipoprotein** composition. The third class is a set of genes implicated directly in the etiology of atherosclerosis, angiogenesis, diabetes, obesity and. . .

DETD . . . genes, CHD1 has been found to bind to a promoter fragment of the HNF4 gene (hepatic nuclear factor 4). Transfection **assays** indicate that CHD1 represses transcription from this promoter suggesting that CHD1 may regulate HNF4 expression in vivo. Pathological consequences of. . .

DETD . . . ligand-activated transcription factors. HNF4 functions as a major regulator of liver-specific gene expression, and is involved in the expression of **apolipoproteins** AI, AII, AIV B and CIII (Kardassis et al., 1996). Mutations in HNF4 have been identified in MODY1 (maturity-onset diabetes. . .

DETD . . . polymerase chain reaction (PCR). This preferred method is exemplified in Example 6. The polymerase chain reaction and other polymerase-driven amplification **assays** can achieve over a million-fold increase in copy number through the use of polymerase-driven amplification cycles. Once amplified, the resulting. . .

DETD As noted above, non-PCR based screening **assays** are also contemplated in this invention. This procedure hybridizes a nucleic acid probe (or an analog such as a methyl. . .

DETD Two-step label amplification methodologies are known in the art. These **assays** work on the principle that a small ligand (such as digoxigenin, biotin, or the like) is attached to a nucleic. . .

DETD . . . example is the biotin-avidin type of interactions. For methods for labeling nucleic acid probes and their use in biotin-avidin based **assays** see Rigby et al., 1977 and Nguyen et al., 1992.

DETD Also within the scope of this invention are the nucleic acid probe **assays** employing a cocktail of nucleic acid probes capable of detecting CHD1. Thus, in one example to detect the presence of. . .

DETD Preferred embodiments relating to methods for detecting CHD1 or its mutations include enzyme-linked immunosorbent **assays** (ELISA), **radioimmunoassays** (RIA), immunoradiometric **assays** (IRMA) and immunoenzymatic **assays** (IEMA), including sandwich **assays** using monoclonal and/or polyclonal antibodies. Exemplary sandwich **assays** are described by David et al. in U.S. Pat. Nos. 4,376,110 and 4,486,530, hereby incorporated by reference, and exemplified in. . .

DETD . . . expressing the CDH1 polypeptide or fragment. Such cells, either in viable or fixed form, can be used for standard binding **assays**, preferably in competitive binding **assays**. One may measure, for example, (a) the formation of complexes formed between a CDH1 polypeptide or fragment and the drug. . .

DETD . . . provides methods of screening for drugs comprising interaction between a drug candidate with a CHD1 polypeptide or fragment thereof and **assaying** (I) for the presence of a complex between the drug candidate and the CHD1 polypeptide or fragment, or (ii) for. . . a ligand such as a polypeptide or DNA sequence, by methods well known in the art. In such competitive binding **assays** the CHD1 polypeptide or fragment is typically labeled. Free CHD1 polypeptide or fragment is separated from that present in a. . .

DETD This invention also contemplates the use of competitive drug screening **assays** in which neutralizing antibodies capable of specifically binding to the CDH1 polypeptide compete with a test compound for binding to. . .

DETD It is also possible to isolate a target-specific antibody, selected by a functional **assay**, and then to solve its crystal structure. In principle, this approach yields a pharmacore upon which subsequent drug design can. . .

DETD . . . the cell is determined. Any metabolic trait of mutant cell lines, such as lipid metabolism or glucose metabolism can be **assayed**. **Assays** for each of these traits are known in the art.

DETD Gel Shift **Assays**. Probes were prepared by PCR amplification of genomic DNA using Pfu and Taq plus long enzymes (Strategene), or by direct. . .

DETD . . . to be involved in lipid metabolism. Several promoter fragments containing these sequences were amplified from genomic DNA and gel shift

assays were performed.

DETD Examples of gel shift **assays** are shown for **ApoAIV**, ApoCIII, ApoE, LPL and LCAT promoter fragments in FIG. 5. GST protein alone does not cause a mobility shift of. . . binding of CHD1.ZnF3-8 to these promoter fragments is sequence specific. Protein dilution experiments indicate that binding of CHD1.ZnF3-8 to the **ApoAIV** fragment has an apparent Kd of approximately 10 nM, an upper limit assuming that all of the partially purified CHD1.ZnF3-8. . . et al., 1992, Kriwacki et al., 1992). Several DNA fragments tested did not bind to CHD1, including sub-fragments of the **ApoAIV** and LPL promoters (FIGS. 5B, 6). These sub-fragments do not possess a CHD1 binding motif, again indicating specificity of binding.. . .

DETD TABLE 12

Genes with binding sites for CHD1 protein

Gene	function	reference
ApoAIV	structural component of HDL	Kardassis, et al., 1996
ApoCIII enhancer	regulates liver expression of **ApoAI**, ApoCIII and **ApoAIV**	Kardassis, et al., 1996
ApoE	structural component of LDL, HDL - binds LDL-R	Davignon, et al., 1988
LPL	**lipoprotein** lipase/interconversion of **lipoproteins**, metabolism of TG-rich **lipoproteins**	Olivercrona, et al., 1993
LCAT	lecithin: cholesterol acyltransferase/metabolism of pre-beta-HDL	Kuivenhoven, et al., 1997
PLTP	phospholipid transport protein/metabolism of pre-beta-HDL	Marques-Vidal, et al., 1997
HTGL	hepatic. . .	

DETD TABLE 13

Positions of promoter fragments that bind CHD1 protein

Gene	GenBank accession #	probe *
ApoAIV	X13368	779-1187
ApoCIII	X13367	675-1052
ApoE	M10065	723-1062
LPL	M29549	199-544
LCAT	X51966	1411-1767
PLTP	U38950	310-446
HTGL	X58779	500-800

. . .

DETD . . . promoter fragments that CHD1 binds to can be grouped into several classes (Table 12). The promoters of a set of **apolipoprotein** (Apo) genes, which encode structural components of circulating **lipoproteins**, comprising the Class 1 genes potentially regulated by CHD1. Class 1 includes the HDL structural proteins **ApoAIV** and ApoE, as well as the ApoCIII enhancer, which regulate the liver specific expression of the **ApoAI**, CIII, AIV genes (reviewed in Kardassis et al., 1996). In all of these promoters the fragments that bind CHD1 have. . . to regulate gene expression in vitro (reviewed in Kardassis et al., 1996--FIG. 7). As described in the background section, the **ApoAI**, ApoCIII, **ApoAIV** loci have been genetically associated with several dyslipidemias and atherosclerosis. ApoE is a component of many circulating **lipoproteins**, and mediates interactions of these proteins with the LDL-receptor. Common polymorphisms of ApoE alter its affinity for the LDL receptor,. . .

DETD The second class of promoters that bind to CHD1 includes several enzymes known to influence **lipoprotein** composition. Class 2 includes the **lipoprotein** lipase gene (LPL), the lecithin:cholesterol acyltransferase gene (LCAT), the phospholipid transport protein gene (PLTP) and the hepatic triglyceride lipase gene. . .

DETD . . . 12, CHD1 has been found to bind to a promoter fragment of the HNF4 gene (hepatic nuclear factor 4). Transfection **assays** indicate that CHD1 represses transcription from this promoter suggesting that CHD1 may regulate HNF4 expression in vivo. Pathological consequences of. . .

DETD . . . ligand-activated transcription factors. HNF4 functions as a major regulator of liver-specific gene expression, and is involved in the expression of **apolipoproteins** AI, AII, AIV B and CIII (Kardassis et al., 1996). Mutations in HNF4 have been identified in MODY1 (maturity-onset diabetes. . .

DETD . . . CHD1 is a sequence specific DNA binding protein. It binds to fragments of the regulatory regions of a subset of **apolipoprotein** genes, a set of genes known to be intimately involved in the regulation of plasma **lipoprotein** metabolism, and a set of genes that have links to atherosclerosis, obesity, NIDDM and insulin resistant syndrome X. CHD1 has. . . shown to bind to the regulatory region of HNF4, whose gene product is involved in regulating the expression of several **apolipoprotein** genes. The binding of CHD1 to these regulatory regions makes it very probable that CHD1 is involved in their regulation,. . .

DETD . . . grown as ascites in mice or in a hollow fiber system to produce sufficient quantities of antibody for characterization and **assay** development.

DETD . . . in larger quantities as biotin conjugates by commercial services. These peptides are used in both solid and solution phase competition **assays** with CHD1 and its interacting partners identified in yeast 2-hybrid screens. Versions of these peptides that are fused to membrane-permeable. . . chemically synthesized, added to cultured cells and the effects on growth, apoptosis, differentiation, cofactor response, and internal changes will be **assayed**.

DETD Sandwich **Assay** for CHD1

DETD . . . amount of bound label, which is proportional to the amount of CHD1 peptide/protein present in the sample, is quantitated. Separate **assays** are performed using monoclonal antibodies that are specific for the wild-type CHD1 as well as monoclonal antibodies specific for each. . .

DETD Two-hybrid **Assay** to Identify Proteins that Interact with CHD1

DETD . . . lacking leucine, tryptophan, and histidine, and containing 25 mM 3-amino-1,2,4-triazole. After one week incubation at 30.degree. C., yeast colonies are **assayed** for expression of the lacZ reporter gene by .beta.-galactosidase filter **assay**. Colonies that both grow in the absence of histidine and are positive for production of .beta.-galactosidase are chosen for further. . .

DETD . . . plasmids encoding various DNA-binding domain fusion proteins, including fusions to CHD1 and human lamin C. Transformants from these experiments are **assayed** for expression of the HIS3 and lacZ reporter genes. Positives that express reporter genes with CHD1 constructs and not with. . .

L8 ANSWER 15 OF 39 USPATFULL
ACCESSION NUMBER: 2000:34393 USPATFULL
TITLE: Systemic inflammatory markers as diagnostic tools in the prevention of atherosclerotic diseases and as tools
to aid in the selection of agents to be used for the prevention and treatment of atherosclerotic disease
INVENTOR(S): Ridker, Paul, Chestnut Hill, MA, United States
Hennekens, Charles H., South Natick, MA, United States
PATENT ASSIGNEE(S): The Brigham and Women's Hospital, Inc., Boston, MA, United States (U.S. corporation)

	NUMBER	KIND	DATE	
PATENT INFORMATION:	US 6040147		20000321	
APPLICATION INFO.:	US 1998-54212		19980402	(9)

DOCUMENT TYPE: Utility
PRIMARY EXAMINER: Saunders, David
LEGAL REPRESENTATIVE: Wolf, Greenfield & Sacks, PC
NUMBER OF CLAIMS: 47
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 7 Drawing Figure(s); 5 Drawing Page(s)
LINE COUNT: 1501
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB The invention involves methods for characterizing an individual's risk profile of developing a future cardiovascular disorder by obtaining a level of the marker of systemic inflammation in the individual. The invention also involves methods for evaluating the likelihood that an individual will benefit from treatment with an agent for reducing the risk of future cardiovascular disorder.

SUMM The invention also contemplates kits comprising a package including an **assay** for a marker of systemic inflammation and instructions, and optionally related materials such as number or color charts, for correlating the level of the marker as determined by the **assay** with a risk of developing a future cardiovascular disorder or with other patient criteria as described above. In important embodiments, the kits also include an **assay** for a cholesterol.

DETD . . . Typically, the level is determined by measuring the level of the marker in a body fluid, for example, blood, lymph, **saliva**, urine and the like. The level can be determined by ELISA, or **immunoassays** or other conventional techniques for determining the presence of the marker. Conventional methods include sending samples of a patient's body. . .

DETD There presently are commercial sources which produce reagents for **assays** for C-reactive protein. These include, but are not limited to, Abbott Pharmaceuticals (Abbott Park, Ill.), CalBiochem (San Diego, Calif.) and. . .

DETD In preferred embodiments the invention provides novel kits or **assays** which are specific for, and have appropriate sensitivity with respect to, predetermined values selected on the basis of the present. . . at particular cut-offs as well as instructions or other printed material for characterizing risk based upon the outcome of the **assay**.

DETD For each case and control, plasma collected and stored at baseline was thawed and **assayed** for C-reactive protein employing enzyme linked immunoabsorbant **assays** (ELISA) based upon purified protein and polyclonal anti-protein antibodies (Calbiochem)..sup.3 In

brief, antibodies are used to coat microtiter plate wells, and biotinylated C-reactive protein plus patient plasma is diluted 1:700 in **assay** buffer (phosphate-buffered saline with 0.1 percent Tween-20, and 1 percent bovine serum albumin). After competition, excess is washed off and. . . Calif.). Purified proteins are then used as standards, with the protein concentrations as determined by the manufacturer. The C-reactive protein **assay** was standardized using the 1st International Reference Standard of the World Health Organization and has sensitivity to 0.08 ug/microliter with standard reference range between 0.5 and 2.5 mg/liter. Methods used to measure total and HDL cholesterol, triglyceride, **lipoprotein**(a), total plasma homocysteine, fibrinogen, D-dimer, and endogenous tissue-type plasminogen activator (tPA) antigen have been described elsewhere..sup.4-8

DETD . . . with the position of the case varied at random within pairs to reduce the possibility of systematic bias and decrease **interassay** variability. The mean coefficient of variation for C-reactive protein across **assay** runs was 4.2 percent.

DETD . . . of coronary artery disease. Similar models were employed to adjust for measured baseline levels of total and HDL cholesterol, triglyceride, **lipoprotein**(a), tPA antigen, fibrinogen, D-dimer, and homocysteine. To evaluate whether aspirin affected these relationships, analyses were repeated for all myocardial infarction. . .

DETD . . . adjusted for body mass index, diabetes, hypertension, a family history of premature coronary artery disease, total cholesterol, HDL cholesterol, triglycerides, **lipoprotein**(a), tPA antigen, D-dimer, fibrinogen, or homocysteine (Table 5).

DETD . . . Triglyceride
Level
Adjusted RR 1.0 1.8 2.1 2.8 <0.001
95% CI -- 1.0-3.2 1.2-3.7 1.6-4.9
p -- 0.06 0.008 <0.001
Lipoprotein(a)
Adjusted RR 1.0 2.0 2.5 2.5 <0.001
95% CI -- 1.2-3.4 1.5-4.2 1.5-4.2
p -- 0.01 <0.001 <0.001
tPA
antigen. . .

DETD . . . time and were not modified by other factors including smoking, body mass index, blood pressure, total and HDL cholesterol, triglyceride, **lipoprotein**(a), tPA antigen, D-dimer, fibrinogen, or homocysteine. In these data, the risk of future myocardial infarction associated with C-reactive protein appears. . .

DETD 4. Stampfer M J, Sacks F M, Salvini S, Willett W C, Hennekens C H. A prospective study of cholesterol, **apolipoproteins**, and the risk of myocardial infarction. N Engl J Med 1991;325:373-81.

DETD 5. Ridker P M, Hennekens C H, Stampfer M J. A prospective study of **lipoprotein**(a) and the risk of myocardial infarction. JAMA 1993;270:2195-2199.

L8 ANSWER 16 OF 39 CAPLUS COPYRIGHT 2001 ACS
ACCESSION NUMBER: 2000:666979 CAPLUS
DOCUMENT NUMBER: 133:219802
TITLE: ELISA method and device for detection of Apo A1, Apo B in **saliva**
INVENTOR(S): Fitzpatrick, Judith; Lenda, Regina B.; Jones, Christopher L.
PATENT ASSIGNEE(S): Serex Inc., USA
SOURCE: PCT Int. Appl., 34 pp.
CODEN: PIXXD2
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1

PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2000055635	A1	20000921	WO 2000-US6810	20000316

W: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

RW: GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG

PRIORITY APPLN. INFO.: US 1999-124562 P 19990316

AB A method has been developed to detect the levels of **apolipoproteins** A-1 and B in **saliva**, which is correlated with the levels of HDL and LDL in serum, resp. In unstimulated **saliva**, the ratio of Apo A to Apo B is correlated with the ratio of HDL to LDL in serum. Albumin can be used to normalize the sample for diln. The high degree of correlation in combination with a simple, quick test that can be performed at the site of collection provides a coast effective, patient friendly means to monitor an individual's risk of heart disease. In the preferred embodiment, **saliva** prodn. is stimulated by means such as breath mint or tart soln. (such as lemon) and the effect of diln. controlled by ref. to albumin. In the most preferred embodiment, the **assay** is an ELISA **assay** performed using the Serex laminated strip format as described in U.S. Patent Nos. 5,710,009, 5,500,375, and 5,451, 504. These strips are advantageous since they serve as the collection and **assay** device.

REFERENCE COUNT: 3

REFERENCE(S): (1) Abbott Lab; WO 9936784 A 1999 CAPLUS

(2) Abbott Lab; WO 9936785 A 1999 CAPLUS

L8 ANSWER 38 OF 39 USPATFULL
ACCESSION NUMBER: 92:96949 USPATFULL
TITLE: Reaction cassette for preforming sequential analytical **assays** by noncentrifugal and noncapillary manipulations
INVENTOR(S): Messenger, Lowry J., Granger, IN, United States
Nelson, Christine D., Edwardsburg, MI, United States
Yip, Kin-Fai, Elkhart, IN, United States
Wogoman, Frank W., Granger, IN, United States
PATENT ASSIGNEE(S): Miles Inc., Elkhart, IN, United States (U.S. corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 5162237		19921110
APPLICATION INFO.:	US 1991-774362		19911008 (7)

DISCLAIMER DATE: 20080205
RELATED APPLN. INFO.: Continuation of Ser. No. US 1989-378039, filed on 11 Jul 1989, now abandoned which is a continuation-in-part of Ser. No. US 1988-179843, filed on 11 Apr 1988, now patented, Pat. No. US 4990075
DOCUMENT TYPE: Utility
PRIMARY EXAMINER: Housel, James C.
ASSISTANT EXAMINER: Redding, David
LEGAL REPRESENTATIVE: Klawitter, Andrew L.
NUMBER OF CLAIMS: 57
EXEMPLARY CLAIM: 18
NUMBER OF DRAWINGS: 12 Drawing Figure(s); 6 Drawing Page(s)
LINE COUNT: 1404
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB Analytical reaction cassette and method for performing sequential analytical **assay** procedures to determine the amount of an analyte in a liquid test mixture. The reaction cassette can be in the form of a substantially square container having a substantially horizontal axis of rotation and incorporated with one or more analytical reagents such that they are contacted with a liquid test mixture in a desired ordered sequence to perform a particular **assay** procedure. Corners provided by the substantially square configuration of the reaction cassette disrupt the flow of liquids disposed in the reaction cassette upon contact therewith to thereby agitate and mix such liquids. A liquid disposed in the reaction cassette is capable of being manipulated and mixed therein by rotating the reaction cassette about the horizontal axis at sufficiently low velocities wherein the transport of such liquid is noncentrifugal and due substantially only to gravitational force. The reaction cassette is particularly useful for performing immunoturbidimetric **assays**, e.g., for the determination of glycated hemoglobin.

TI Reaction cassette for preforming sequential analytical **assays** by noncentrifugal and noncapillary manipulations

AB Analytical reaction cassette and method for performing sequential analytical **assay** procedures to determine the amount of an analyte in a liquid test mixture. The reaction cassette can be in the. . . reagents such that they are contacted with a liquid test mixture in a desired ordered sequence to perform a particular **assay**

procedure. Corners provided by the substantially square configuration of the reaction cassette disrupt the flow of liquids disposed in the. . . such liquid is noncentrifugal and due substantially only to gravitational force. The reaction cassette is particularly useful for performing immunoturbidimetric **assays**, e.g., for the determination of glycated hemoglobin.

SUMM The present invention relates to analytical **assay** procedures for determining the amount of an analyte present in a test sample involving liquid analytical reactions between the analyte. . .

SUMM . . . of a liquid reaction mixture and which, in most instances, must be performed sequentially in order to carry out an **assay** protocol. Manipulations such as pipetting, mixing and agitation, periods of incubation, centrifugation, separation steps, and the like, are subject to. . . some instances, such devices nevertheless still require a number of manual manipulative steps during the course of carrying out an **assay** protocol, particularly for performing sample transfer and mixing steps.

SUMM . . . side thereof which defines a conduit and a plurality of reagent reservoirs isolated from one another by frangible seals. An **assay** tube is situated at one end of the conduit and a sample injection reservoir is situated at the other end. . . frangible seals and force the reagents from their respective reservoirs into the conduit for the purpose of carrying out the **assay**.

SUMM Another object of the present invention is to provide a device for performing sequential analytical **assay** procedures which does not rely upon centrifugal or capillary movement of liquids.

SUMM Still another object of the present invention is to provide a device for performing sequential analytical **assay** procedures which requires a minimal number of manipulation steps and which is easy to manipulate and operate.

SUMM Still further, another object of the present invention is to provide a device for performing sequential analytical **assay** procedures which is easily adaptable to a physician's office or a small clinical laboratory.

SUMM The present invention provides a self-contained reaction cassette or vessel and a method for performing analytical **assay** procedures involving sequential analytical reactions in a liquid test mixture between an analyte and one or more analytical reagents which react with the analyte to produce a detectable signal. The device is particularly useful for performing **immunoassays** which typically require a number of mixing steps, as well as other cumbersome manipulative steps, such as pipetting and incubation. . . of a liquid mixture to zones or areas in the device designed for performing the various functional steps of the **assay**, and (b) oscillation of the device to agitate the liquid mixture in contact with flow disrupting means, such as a. . .

SUMM . . . the device is incorporated with one or more of the necessary analytical reagents for carrying out a particular sequential analytical **assay** procedure. A liquid test mixture is formed in the device and can be sequentially contacted and reacted with the analytical. . . liquid mixtures agitated and mixed at relatively low velocities, without the need for additional external manipulative steps to complete the **assay**. The device also permits the convenient measurement of the detectable response produced by the analytical reactions between the

analyte and. . . the analytical reagents and, where one or more detectable responses are produced subsequent to or during the performance of the **assay**, the device can be easily manipulated to permit the convenient measurement thereof in the device during the course of the **assay**.

SUMM . . . being manipulated in order to introduce the liquid reagent into the reaction channel during the course of performing an analytical **assay** procedure. Preferably, the liquid delivery means is in the form of a reservoir body closed by an externally manipulable, removable,. . .

SUMM . . . relatively slowly about the horizontal axis. Accordingly, once a liquid test mixture has been formed in the device, an analytical **assay** procedure, including the agitation and mixing of the liquid test mixture with the analytical reagents, can be carried out by.

. .

SUMM An analytical **assay** procedure employing the device of the present invention in general terms is performed by introducing a test sample into the. . .

SUMM . . . reaction channel, and a liquid reagent is contained in a centrally located liquid delivery means as described above. An analytical **assay** procedure is carried out by introducing a test sample into the reaction channel and introducing the liquid reagent into the reaction channel. It is to be understood that depending upon the particular **assay** protocol, the liquid reagent can be introduced and mixed with the test sample either prior to contacting the liquid test. . .

SUMM . . . be described in greater detail hereinafter, the device of the present invention is not intended to be limited to analytical **assay** procedures as described above, but can be employed to carry out substantially any sequential analytical **assay** procedure involving a number of manipulative steps in a desired ordered sequence and a plurality of analytical **assay** reagents. In addition, the open liquid flow communication provided along the reaction channel permits the measurement of one or more. . . gravity along the reaction channel to one or more viewing zones for multiple measurements during the course of a single **assay**.

DETD . . . into device 10 in order to prevent liquid loss when manipulating device 10 during and after the course of an **assay**.

DETD . . . 10 further includes a liquid delivery reservoir 30 adapted to contain a buffer and/or liquid reagent for performing an analytical **assay** procedure, preferably from between about 0.25 mL and about 10 mL, more preferably from between about 0.4 mL and about. . .

DETD . . . a liquid therealong and to substantially prevent surface tension or other physical phenomena from occurring during the course of an **assay** procedure. Such treatment of the surfaces includes, but is not intended to be limited to, plasma treatments such as plasma.

. .

DETD . . . the present invention. Accordingly, a particularly preferred embodiment of the present invention and its use in performing a sequential analytical **assay** procedure will now be described in order to provide a better understanding of the present invention.

DETD . . . which includes a capillary sampling tube 60. The liquid capacity of capillary tube 60 will depend upon the particular analytical **assay** procedure which is to be performed in device 40, and, accordingly, will vary in size to introduce a predetermined amount. . . example, a plug member (not shown) or other means to prevent the loss of liquid during the course of an **assay**.

DETD . . . third and fourth corners 52, 54 and 55, respectively, and are incorporated with analytical reagents for performing a particular analytical **assay** procedure. The analytical reagents are preferably present in the reagent zones in a substantially dry, water soluble, suspendable, or dissolvable. . .

DETD . . . or direction of rotation of device 40. For example, although three reagent zones 61, 62 and 63 are shown, other **assay** procedures can also be performed in device 40 with the number of analytical reagents will of course depending upon the particular **assay** requirements. Furthermore, the device can include less than the required number of analytical reagents for performing an analytical **assay** procedure where one or more reaction mixtures thereof can first be formed outside of the device and then introduced into the device to complete the **assay**.

DETD . . . provides a first detectable response or measurable characteristic which is required or desired to be measured according to a particular **assay** protocol, device 40 is rotated in a clockwise direction in order that first reaction mixture 71 is transported by gravity. . . (FIG. 5e). Any such first detectable response provided by first reaction mixture 71 can then be measured, and

the remaining **assay** steps carried out subsequent thereto. For example, such first detectable response can be a total hemoglobin measurement where the liquid test sample is a whole blood sample, such as when performing an **assay** for the percent of glycated hemoglobin in a whole blood sample, as will be described in greater detail hereinafter.

DETD . . . a stationary position to incubate third reaction mixture 73 as described above. Typically, the final reaction mixture in an analytical **assay** procedure, in this case third reaction mixture 73, will provide a detectable response which is measured and correlated to the. . .

DETD Device 40 can be used to perform turbidimetric and nephelometric **assays** in general which are known in the art to determine analytes of interest in a variety of test samples, particularly biological fluids such as whole blood, serum, plasma, urine, **saliva**, cerebrospinal fluid, and the like. For example, agglutination **immunoassays** and agglutination inhibition **immunoassays** can be performed wherein the analytical reagents thereof are incorporated into reaction channel 49 in the desired order sequence to. . .

DETD In particular, device 40 according to the present invention is useful for performing an immunoturbidimetric **assay** for determining hemoglobin Alc (HbAlc), a glycated hemoglobin derivative. According to such **assay**, hemoglobin in a whole blood sample is converted into a denatured thiocyan-met-hemoglobin form which serves as the basis for first measuring total sample hemoglobin, and then measuring the denatured HbAlc form by **immunoassay**. The **immunoassay** is based on the specific interaction of an antibody particle reagent and

an agglutinator reagent, such as described by U.S.. . .

DETD . . . latex). Such latex particles which are useful will be evident to the worker familiar with the field of latex agglutination **immunoassays**. In general, such particles require the properties necessary to serve as a stable support for the desired antibody reagent for the **assay** and to undergo agglutination in the presence of an agglutinator reagent sufficient for analytical purposes. Latex particles are prepared generally. . .

DETD . . . epitopic binding sites for the antibody reagent and can be prepared according to techniques familiar to the field of agglutination **immunoassays**. This reagent will, in general terms, comprise a plurality of epitopic binding sites for the anti-analyte antibody reagent. Such sites. . . analyte itself or a suitable analog that retains sufficient capacity to be bound by the antibody for purposes of an **assay**. Such analog can, in the case of a protein analyte, comprise a suitable fragment, prepared synthetically or by digestion,

comprising. . .

DETD The aforementioned reagents can be incorporated into device 40 in order that an immunoturbidimetric **assay** for HbAlc can be performed therein substantially as described above and as shown in FIGS. 5a-5h. In particular, reagent zone. . .

DETD Device 40 is also useful for performing an immunometric **assay** involving binding among the analyte, a labeled reagent comprising an anti-analyte antibody reagent labeled with a detectable chemical group, and an immobilized form of the analyte or a binding analog thereof. According to such **assay**, the amount of labeled antibody reagent bound to the analyte from the liquid test sample or to that which is. . .

DETD . . . can be any material having a detectable physical or chemical property. Such materials have been well-developed in the field of **immunoassays** and in general any label useful in such methods can be applied to such immunometric **assay** procedures. For example, such chemical groups having detectable physical properties are those groups which are detected on the basis of. . .

DETD . . . fields without becoming permanently magnetized, which would otherwise result in the undesirable magnetic aggregation thereof during the performance of an **immunoassay**.

DETD As will be understood by one skilled in the art apprised of the foregoing considerations, an immunometric **assay** employing the device can be performed by incorporating such immunometric **assay** reagents along reaction channel 49 as described above. It is to be understood that where the device 40 is employed to perform such immunometric **assay**, a buffer reagent, diluent, or the like can be contained by liquid delivery reservoir 30 and introduced into reaction channel. . . group, such as a chromogenic substrate for the enzyme, and introduced into reaction channel 49 at the end of the **assay** to interact with the enzyme component of the labeled reagent to provide the detectable response.

DETD The **assay** involved was a turbidimetric **immunoassay** for HbAlc as described above. The degree of mixing in the various cassettes was assessed by measuring the precision of the **immunoassay** conducted in the cassettes. Thorough mixing eliminated the time dependence and irreproducibility factor from the **immunoassay** resulting in good precision of measurement. For the Round and Square Cassettes, the antibody-latex and agglutinator reagents were dispensed in. . .

DETD In carrying out **immunoassays** in the Square and Round Cassettes, approximately 0.5 mL of denaturant solution was added to the device and mixed with a blood test sample and the **immunoassay** reagents in accordance with the respective operational methods for the two types of devices. For the Control Cuvettes, the denaturant solution was first added to the device and mixed with the test sample. Thereafter, the **immunoassay** reagents were added in the form of a thoroughly mixed liquid solution. The agglutination response in all cassettes were then. . .

DETD . . . disrupting means in accordance with the present invention provides significant improvement in mixing and thus in the precision of the **assay** (Square Cassette v. Round Cassette), and that the precision thereby attained is roughly identical with that attained when premixed liquid. . .

DETD . . . will be liquid in nature, and will include biological liquids such as blood, serum, plasma, urine, cerebral fluid, spinal fluid, **saliva**, swab extracts, sputum, and so forth. Non-biological specimens can also be processed such as industrial, food, and environmental liquids. A. . .

DETD The analytical **assay** procedures described above can be used in the determination of a variety of analytes. The analyte usually is a peptide,. . .

DETD Representative protein analytes include the classes of protamines, mucoproteins, glycoproteins, globulins, albumins, scleroproteins,

phosphoproteins, histones, **lipoproteins**, including, but not intended to be limited to, **apolipoproteins** such as **apolipoprotein**-AI and **apolipoprotein**-B100, chromoproteins, and nucleoproteins. Examples of specific proteins are prealbumin, .alpha.-**lipoproteins**, human serum albumin, .alpha.-acid glycoprotein, .alpha..sub.1 -antitrypsin, .alpha..sub.1 -glycoprotein, transcortin, thyroxine binding globulin, haptoglobin, hemoglobin, glycated peptide sequence in the beta-subunit of human hemoglobin, myoglobulin, ceruloplasmin, .alpha..sub.2 -macroglobulin, .beta.-**lipoprotein**, erythopoietin, transferrin, hemopexin, fibrinogen, the immunolobulins such as IgG, IgM, IgA, IgD, and IgE, and their fragments, e.g., Fc and. . .

DETD . . . further to be understood that the device of the present invention is not intended to be limited to performing such **assay** procedures as specifically described above, but can also be incorporated with analytical reagents for performing a variety of other **assay** procedures known in the art. For example, such other **assay** procedures include, but are not intended to be limited to, apoenzyme reactivation **immunoassay** system (ARIS) as described by U.S. Pat. No. 4,238,565; substrate labeled fluorescent **immunoassay** (SLFIA) as described by U.S. Pat. No. 4,279,992; enzyme inhibitor-labeled **immunoassay** as described by U.S. Pat. No. 4,134,792; enzyme multiplied **immunoassay** technique (EMIT.RTM.) as described by U.S. Pat. Nos. 3,817,837 and 4,043,872; cloned enzyme donor **immunoassay** (CEDIA.RTM.) as described in U.S. Pat. No. 4,708,929; and fluorescence polarization **immunoassay** (TDX.RTM.) as described by U.S. Pat. No. 4,510,251; and the like.

DETD . . . contact plate type having flexpoint connections, for heating a liquid test sample or reaction mixtures when required by a particular **assay** protocol, and optical sensors for properly positioning the device in the mechanical device. Preferably, the various mechanical and electronic components. . . including, for example, a slot or opening for receiving the device or, where it is desired to simultaneously perform an **assay** protocol on more than one liquid test samples, more than one of the device of the present invention.

DETD . . . the cassette for the purpose of absorbing the full volume of liquid mixture in the device upon completion of the **assay**. In this way, no free flowing liquid would remain in the device at the time of disposal thus lessening the. . . The absorbent mass would be placed, for example, at one end of the reaction channel and upon completion of the **assay** procedure, the device would be rotated to bring the liquid mixture into contact with the absorbent. For instance, such an. . .

L11 ANSWER 6 OF 15 USPATFULL

ACCESSION NUMBER:	2000:1709 USPATFULL
TITLE:	Determination of analytes in biological fluids in the presence of substances interfering with **assays** therefor
INVENTOR(S):	Ollington, James F., Chelmsford, MA, United States Byrnes, Ronald J., West Bridgewater, MA, United States Pogorzelski, Donald E., Nashua, NH, United States
PATENT ASSIGNEE(S): (U.S. corporation)	Genzyme Corporation, Cambridge, MA, United States

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 6010866		20000104
APPLICATION INFO.:	US 1995-393337		19950223 (8)

RELATED APPLN. INFO.:	Division of Ser. No. US 1990-515596, filed on 27 Apr 1990, now patented, Pat. No. US 5403745
DOCUMENT TYPE:	Utility
PRIMARY EXAMINER:	Chin, Christopher L.
LEGAL REPRESENTATIVE:	Bromberg & Sunstein LLP
NUMBER OF CLAIMS:	7
EXEMPLARY CLAIM:	1
NUMBER OF DRAWINGS:	20 Drawing Figure(s); 13 Drawing Page(s)
LINE COUNT:	862

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB A method is provided, in one embodiment, for the determination of an analyte in a biological fluid sample in the presence of a substance interfering with an **assay** for the analyte. This embodiment is implemented by using antibodies to cause the selective immunoreaction of at least one of the analyte or the interfering substance and then conducting an **assay** for the analyte in at least one of the immunoreactants or the non-reactants. Another embodiment provides a disposable reaction device to implement the method. The invention is applicable to the detection of a wide variety of analytes, including cholesterol in a targeted lipoprotein class in the presence of cholesterol in another class; to targeted isozymes of enzymes such as creatine kinase, lactate dehydrogenase, amylase, and alkaline or acid phosphatases in the presence of other isozymes; as well as to targeted immunoglobulins in the presence of non-targeted immunoglobulins.

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L11 ANSWER 6 OF 15 USPATFULL

TI Determination of analytes in biological fluids in the presence of substances interfering with **assays** therefor

AB . . . for the determination of an analyte in a biological fluid sample in the presence of a substance interfering with an **assay** for the analyte. This embodiment is implemented by using antibodies to cause the selective immunoreaction of at least one of the analyte or the interfering substance and then conducting an **assay** for the analyte in at least one of the immunoreactants or the non-reactants. Another embodiment provides a disposable reaction device. . .

SUMM The present invention relates to the determination of analytes in the presence of substances interfering with **assays** for such

analytes. The invention also relates to the use of devices for implementing such determination. The invention can be. . .

SUMM The efficacy of **assays** for analytes in a biological fluid sample can be reduced due to the presence of substances which interfere with the **assay** (Kaplan, L. and Pesce, A., "Interferences in Chemical Analysis" in Clinical Chemistry (Kaplan, L. and Pesce, A., eds., The C.. . . (1989)). For example, compounds such as hemoglobin or bilirubin, which have a strong visible absorbance, can interfere with a spectrophotometric **assay** for an analyte. Kaplan and Pesce, id.

SUMM . . . of the art in the detection of analytes in biological fluids. The prior art known to the inventors lacks an **assay**, for cholesterol in specific lipoprotein classes, that is simultaneously (i) easily interpretable from an epidemiological point of view; (ii) easily,. . . inexpensively implemented, and (iii) universally applicable to all routine clinical chemistry testing systems. Indeed, the inventors are unaware of any **assays**, for specific classes of an analyte that are the subject of the routine tests described above, that meets these two. . .

SUMM . . . LDL cholesterol and decreased levels of HDL cholesterol have been shown to be risk factors for CHD. Consequently, clinical diagnostic **assays** for cholesterol content in the major lipoprotein classes are performed extensively and a large body of statistical data on the. . .

SUMM In the clinical laboratory, the following **assays** are performed routinely to characterize the lipid and cholesterol profile of a plasma or serum sample: (i) Triglycerides are determined. . .

SUMM In research laboratories a variety of immuno-based analytical techniques have been employed to quantitate lipoproteins, including radial immunodiffusion, **radioimmunoassay** and **electroimmunoassay**, but these techniques are too cumbersome to be employed in a clinical diagnostic setting where large numbers of samples must be handled rapidly. This disadvantage may be addressed by using an enzyme-linked immunoabsorbant **assay** (ELISA), and this is an area of active investigation (Ordovas et. al., J. Lipid Research 28:1216-1224 (1987)).

SUMM . . . known. Longenecker (U.S. Pat. No. 4,302,536 (1981)) reported the determination of antigenic materials in biological fluids and cells by calorimetric **immunoassay** with an adduct of antibody and chromo-protein. Onishi and Ito (Eur. Pat. No. 327,918 (1989)) reported an **immunoassay** using the homogeneous competitive reaction between a target and labelled substance and a specific binder. Freytag and Ishikawa (U.S. Pat. No. 4,657,853 (1987)) reported a high sensitivity **immunoassay** using a polymeric enzyme-antibody conjugate. Nippon (Jap. Pat. No. 59226864 (1984)) reported an **immunoassay** in which levels of transforming growth factor (TGF) in a liquid sample are detected using an immobilized TGF antibody and an enzyme labelled TGF antibody. Gomez and Wicks (U.S. Pat. No. 4,353,982 (1982)) report an **immunoassay** for creatine kinase in blood serum using iodine-125 labelled antibody to precipitate immune complex mixtures.

SUMM . . . followed by cholesterol quantitation in the lipoprotein class remaining in solution. Heuck et al. reported the use of antibodies to **ApoB** to precipitate LDL and VLDL followed by measuring cholesterol levels in the HDL left in the supernatant. Antibodies to **apoAI** and apoc were also used, to precipitate HDL and VLDL, followed by determination of cholesterol levels in the LDL left. . .

SUMM . . . a method for detecting an analyte in a biological fluid sample in the presence of a substance interfering with an **assay** for the analyte. This embodiment is implemented by using antibodies to cause

the selective immunoprecipitation of at least one of the analyte or the interfering substance and then conducting an **assay** for the analyte in at least one of the immuno-reactants or the non-reactants. In another embodiment, the invention provides a. . .

DRWD FIG. 4 is a graph showing the time course for the precipitation of human plasma **ApoB**-containing lipoprotein particles by freeze-dried anti-ND-LDL sera as determined by supernatant cholesterol estimation, in accordance with Example 1.

DRWD FIG. 6 is a graph supernatant cholesterol and **ApoB** levels in accordance with Example 1.

DETD . . . for the detection of an analyte in a biological fluid sample, in the presence of a substance interfering with an **assay** for such analyte, using immunoseparation technology. In the invention, antibodies are used to cause selective immunoreaction of the analyte or a substance interfering with an **assay** for the analyte, and then the analyte is detected by **assay** of one of either the immunoreactants or the non-reactants. In one embodiment of the invention, a reaction device is used. . . linked dextran, cross linked polysaccharides, or cross linked acrylamide, microporous filters or membranes. Other suitable insoluble carriers include a coiled **strip** or the interior wall of the reaction device itself. Antibodies may also be bound to soluble large MW polymers to. . .

DETD . . . in accordance with a preferred embodiment of the invention, may be directly related to the national reference system. (Other immuno-based **assays** currently provided by embodiments of the invention include apoliproprotein quantitation, for which statistically significant clinical data, i.e., normal ranges in. . .

DETD . . . for characteristics relevant to other immuno-based techniques, e.g., good binding to plastic surfaces as needed for ELISA procedures. In addition, **assay** in accordance with this invention may be implemented with a device containing stabilized antibodies, which allows rapid, inexpensive, and efficient. . .

DETD Starting with purified ND-LDL, **ApoB** was isolated by electrophoresis through a preparative 15% polyacrylamide gel followed by excision of the separated **ApoB** band. Upon completion of electrophoresis the gel was stained with sodium acetate to visualize protein bands, as described in E. Harlow and D. Lane, editors, Antibodies. A Laboratory Manual (Cold Spring Harbor Press, 1988). The **ApoB** containing gel region was excised using a scalpel or razor and the polyacrylamide gel was homogenized by repeated passages though progressively narrower gauge needles according to the method described essentially in Antibodies, ibid. The **ApoB** in homogenized acrylamide may be stored at@4.degree. C. prior to immunization.

DETD Starting with approximately 10 ml purified ND-HDL, **ApoAI** and **ApoAII** were purified by chromatography over Sephacryl S-200 essentially as described by Brewer, et al. (1986), Methods in Enzymology 128:223-246. Fractions containing separated **ApoAI** and AII were quantitated for protein by Biorad **assay** and electrophoresed through a preparative 15% polyacrylamide gel under denaturing conditions. Protein bands were visualized, excised and prepared for immunization. . .

DETD It is within the scope of the invention to use purified **ApoAI** and **ApoAII** to immunize individual goats, and obtain the corresponding antisera.

DETD . . . be free of material cross-reactive to any of the apoproteins of purified HDL. The ND-LDL antisera showed cross-reactivity only to **ApoB** in VLDL, LDL, and whole plasma.

DETD The anti-narrow density HDL sera showed reactivity to **ApoAI** in

HDL and whole plasma and reactivity to ApoCs and ApoE in HDL, VLDL and whole plasma. A small amount of cross-reactive material to **ApoB** was detected and can be removed by immunoaffinity chromatography over an

ND-HDL-sepharose column. The column is prepared in the following. . .

DETD The filtrate was **assayed** for total cholesterol using an Abbott ACA 200 chemistry analyzer and Abbott A-Gent cholesterol reagents, although any cholesterol determination method. . .

DETD Apoprotein **immunoassays** were used to evaluate the comparative specificity of lipoprotein particle separation between the immunoseparation method and the dextran sulfate method.. . . particles by anti-ND-LDL sera monitored by the reduction in supernatant (filtrate) cholesterol values (as described above) and by Apoprotein B **immunoassay** measured by the method of Ordovas, et al. (1987). At the equivalent of 350 .mu.l anti-ND-LDL sera, the supernatant cholesterol value was 32.9 mg/dl, the dextran sulfate control value was 30.0 mg/dl. By **immunoassay**, the following results were obtained: at 350 .mu.l of antisera the supernatant **ApoB** level was undetectable above the background control; the dextran sulfate supernatant yielded 1.2 mg/dl **ApoB** remaining in the supernatant.

DETD . . . its base, and (iii) an immobilized antibody component 76 which may, for example, be antibody-coated, or beads or a coiled **strip**, such as represented in FIG. 8), which is contained within the reaction

chamber 74. Any other suitable means for immobilization. . .

DETD . . . a screw cap gripping the threaded shoulder 73 in FIG. 10) or the filtrate or an aliquot thereof may be **assayed** for non-reactant analyte(s), using routine **assays**.

DETD . . . at its bottom. The reaction pipette 114 contains prepacked aliquots of antibodies immobilized, for example, on beads or a coiled **strip** (FIG. 13), or otherwise in the manner as discussed above in connection with FIG. 8. The reaction pipette 114 may. . .

DETD . . . the operation of the embodiment described above in connection with FIGS. 11-17. The collected filtrate or immunoreactants can then be **assayed** using routine tests.

DETD . . . through 20 must be antibodies to one of the targeted analyte or

to the non-targeted analyte, depending on how the **assay** is conducted following the immunoseparation. The antibodies may be prepared

using methods known in the art.

DETD . . . routine test for the class of analytes, then following separation in accordance with the invention, the targeted analyte can be

assayed using the routine test for the class of analytes. For example, one may use a routine test for amylase to. . . separation, for example, using antibodies to all isozymes of amylase other than pancreatic specific amylase. Thereafter the filtrate may be **assayed** using the routine test to identify the level of pancreatic specific amylase in the sample. A similar strategy may be used to **assay** any targeted analyte in a class of analytes for which a routine test exists.

DETD . . . invention may also be used to remove substances such as bilirubin and hemoglobin that can interfere with spectrophotometric or other **assays** for an analyte. In such instances, antibodies to bilirubin and hemoglobin may be employed to achieve their immunoseparation (using the invention) from the sample prior to conduct of an **assay** in accordance with prior art techniques.

CLM What is claimed is:

1. A disposable reaction device for separating an analyte in a biological fluid, from a substance interfering with an **assay** for the analyte, comprising: (i) a reaction chamber into which the biological fluid may be placed, the reaction chamber including. . .

2. A device according to claim 1, wherein the **assay** is applicable to a class of molecules, the analyte is one type of molecule

in the class, and the interfering. . .

(FILE 'HOME' ENTERED AT 13:58:18 ON 25 JUN 2001)

FILE 'MEDLINE, EMBASE, SCISEARCH, USPATFULL, CAPLUS' ENTERED AT 13:59:18 ON 25 JUN 2001

L1	6312	S (APOLIPOPROTEIN? AND LIPOPROTEIN?) AND ?ASSAY?
L2	348	S ((APO!A? OR APOA?) AND (APO!B? OR APOB?)) AND ?ASSAY?
L3	279	S L1 AND L2
L4	12	S L3 AND ((DRY CHEMI?) OR ?STRIP? OR DIPSTICK?)
L5	12	DUP REM L4 (0 DUPLICATES REMOVED)
L6	39	S L1 AND SALIVA
L7	1	S L6 AND L2
L8	39	DUP REM L6 (0 DUPLICATES REMOVED)
L9	2	S L2 AND SALIVA?
L10	15	S L2 AND ((DRY CHEMI?) OR ?STRIP? OR DIPSTICK?)
L11	15	DUP REM L10 (0 DUPLICATES REMOVED)
L12	1	S L11 AND SALIVA?
L13	349	S (APOLIPOPROTEIN? OR LIPOPROTEIN?) (6P) (SALIVA?)
L14	2	S ((APO!A? OR APOA?) AND (APO!B? OR APOB?)) AND SALIVA?